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DOUTORAMENTO EM MEDICINA

ANKYLOSING SPONDYLITIS: genomic and functional characterization of candidate genes and their repercussion in clinical practice

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"Há um tempo em que é preciso abandonar as roupas usadas, que já têm a forma do nosso corpo, e esquecer os nossos caminhos, que nos levam sempre aos mesmos lugares. É o tempo da travessia: e, se não ousarmos fazê-la, teremos ficado, para sempre, à margem de nós mesmos".

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Existem muitos momentos importantes que pincelam de cores várias o quadro das nossas vidas… Os anos recentes, de 2007-2011, criaram uma mancha não formatável de tons não definíveis… Um *mix* de pessoas, lugares, experiências, emoções estão aqui representados. Momentos de sorriso rasgado, de movimentos incontroláveis incapazes de esconder uma vitória,… momentos de fácies enrugado, carregado de tons cinza, de desespero, de desistência,… momentos de relançamento, de persistência, de se acreditar no progresso…

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RESUMO

Introdução: A espondilite anquilosante (EA) é uma doença inflamatória crónica caracterizada pela inflamação das articulações sacroilíacas e da coluna. A anquilose progressiva motiva uma deterioração gradual da função física e da qualidade de vida. O diagnóstico e o tratamento precoces podem contribuir para um melhor prognóstico. Neste contexto, a identificação de biomarcadores, assume-se como sendo muito útil para a prática clínica e representa hoje um grande desafio para a comunidade científica.

Objetivos: Este estudo teve como objetivos: 1 - caracterizar a EA em Portugal; 2 - investigar possíveis associações entre genes, MHC e não-MHC, com a suscetibilidade e as características fenotípicas da EA; 3 - identificar genes candidatos associados a EA através da tecnologia de *microarray*.

Material e Métodos: Foram recrutados doentes com EA, de acordo com os critérios modificados de Nova Iorque, nas consultas de Reumatologia dos diferentes hospitais participantes. Colecionaram-se dados demográficos, clínicos e radiológicos e colhidas amostras de sangue periférico. Selecionaram-se de forma aleatória, doentes *HLA-B27* positivos, os quais foram tipados em termos de HLA classe I e II por PCR-rSSOP. Os haplótipos HLA estendidos foram estimados pelo algoritmo *Expectation Maximization* com recurso ao *software* Arlequin v3.11. As variantes alélicas dos genes *IL23R, ERAP1* e *ANKH* foram estudadas através de ensaios de discriminação alélica TaqMan. A análise de associação foi realizada utilizando testes da *Cochrane-Armitage* e de regressão linear, tal como implementado pelo *PLINK*, para variáveis qualitativas e quantitativas, respetivamente. O estudo de expressão génica foi realizado por *Illumina HT-12 Whole-Genome Expression BeadChips.* Os genes candidatos foram validados usando *qPCR-based TaqMan Low Density Arrays (TLDAs).*

Resultados: Foram incluídos 369 doentes (62,3% do sexo masculino, com idade média de 45,4 ± 13,2 anos, duração média da doença de 11,4 ± 10,5 anos). No momento da avaliação, 49,9% tinham doença axial, 2,4% periférica, 40,9% mista e 7,1% entesopática. A uveíte anterior aguda (33,6%) foi a manifestação extra-articular mais comum. Foram positivos para o *HLA-B27*, 80,3% dos doentes. Os haplótipo *A*02/B*27/Cw*02/DRB1*01/DQB1*05* parece conferir suscetibilidade para a EA, e o *A*02/B*27/Cw*01/DRB1*08/DQB1*04* parece conferir proteção em termos de atividade, repercussão funcional e radiológica da doença. Três variantes (2 para *IL23R* e 1 para *ERAP1*) mostraram significativa associação com a doença, confirmando a associação destes genes com a EA na população Portuguesa. O mesmo não se verificou com as variantes estudadas do *ANKH*. Não se verificou associação entre as variantes génicas não-MHC e as manifestações clínicas da EA. Foi identificado um perfil de expressão génica para a EA, tendo sido validados catorze genes - alguns têm um papel bem documentado em termos de inflamação, outros no metabolismo da cartilagem e do osso.

Conclusões: Foi estabelecido um perfil demográfico e clínico dos doentes com EA em Portugal. A identificação de variantes génicas e de um perfil de expressão contribuem para uma melhor compreensão da sua fisiopatologia e podem ser úteis para estabelecer modelos com relevância em termos de diagnóstico, prognóstico e orientação terapêutica dos doentes.

ABSTRACT

Background: Ankylosing Spondylitis (AS) is a chronic inflammatory disorder characterized by inflammation in the spine and sacroiliac joints leading to progressive joint ankylosis and in progressive deterioration of physical function and quality of life. An early diagnosis and early therapy may contribute to a better prognosis. The identification of biomarkers would be helpful and represents a great challenge for the scientific community.

Objectives: The present study had the following aims: 1- to characterize the pattern of AS in Portuguese patients; 2- to investigate MHC and non-MHC gene associations with susceptibility and phenotypic features of AS and; 3- to identify candidate genes associated with AS by means of whole-genome microarray.

Material and Methods: AS was defined in accordance to the modified New York criteria and AS cases were recruited from hospital outcares patient clinics. Demographic and clinical data were recorded and blood samples collected. A random group of *HLA-B27* positive patients and controls were selected and typed for HLA class I and II by PCR-rSSOP. The extended HLA haplotypes were estimated by Expectation Maximization Algorithm using Arlequin v3.11 software. Genotyping of *IL23R*, *ERAP1* and *ANKH* allelic variants was carried out with TaqMan allelic discrimination assays. Association analysis was performed using the Cochrane-Armitage and linear regression tests as implemented in PLINK, for dichotomous and quantitative variables, respectively. Gene expression profile was carried out using Illumina HT-12 Whole-Genome Expression BeadChips and candidate genes were validated using qPCR-based TaqMan Low Density Arrays (TLDAs).

Results: A total of 369 patients (62.3% male; mean age 45.4±13.2 years; mean disease duration 11.4±10.5 years), were included. Regarding clinical disease pattern, at the time of assessment, 49.9% had axial disease, 2.4% peripheral disease, 40.9% mixed disease and 7.1% isolated enthesopathic disease. Acute anterior uveitis (33.6%) was the most common extra-articular manifestation. 80.3% of AS patients were *HLA-B27* positive. The haplotype *A*02/B*27/Cw*02/DRB1*01/DQB1*05* seems to confer susceptibility to AS, whereas *A*02/B*27/Cw*01/DRB1*08/DQB1*04* seems to provide protection in terms of disease activity, functional and radiological repercussion. Three markers (two for *IL23R* and one for *ERAP1*) showed significant single-locus disease associations. Association of these genes with AS in the Portuguese population was confirmed, whereas *ANKH* markers studied did not show an association with AS. No association was seen between non-MHC genes and clinical manifestations of AS. A gene expression signature for AS was established; among the fourteen validated genes, a number of them have a well-documented inflammatory role or in modulation of cartilage and bone metabolism.

Conclusions: A demographic and clinical profile of patients with AS in Portugal was established. Identification of genetic variants of target genes as well as gene expression signatures could provide a better understanding of AS pathophysiology and could be useful to establish models with relevance in terms of susceptibility, prognosis, and potential therapeutic guidance.

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ABBREVIATIONS AND SYMBOLS

AAU - Acute Anterior Uveitis

APC - Adenomatous Polyposis Coli

ANKH - Ankylosis, Progressive Homolog (mouse)

ANTXR2 - Anthrax Toxin Receptor 2

AS - Ankylosing Spondylitis

ASAS - Assessment of SpondyloArthritis International Society

ASDAS - Ankylosing Spondylitis Disease Activity Score

AUC - Area Under the Curve

BASDAI - Bath Ankylosing Spondylitis Disease Activity Index

BASFI - Bath Ankylosing Spondylitis Functional Index

BASMI - Bath Ankylosing Spondylitis Metrology Index

BiP - Binding immunoglobulin Protein

BMP - Bone Morphogenic Protein

CARD9 - Caspase Recruitment Domain family, member 9, gene

CD - Crohn's Disease

CI - Confidence Interval

CK1 α - Casein Kinase 1α

CLEC - C-type Lectin-like receptor, gene

CLEC4D - C-type Lectin Domain family 4, member D, gene

CLRs - C-type Lectin Receptors

COL1A - Pro-alpha2 Chain of type I collagen

CRD - Carbohydrate Recognition Domain

CRP - C-Reactive Protein

CTNNAL1 - Catenin (cadherin-associated protein) alpha-like 1, gene

CYP2D6 - Cytochrome P450 2D6 gene

CX3CL1 - Chemokine (C-X3-C motif) ligand 1, gene

CX3CR1 - Chemokine (C-X3-C motif) receptor 1, gene

CXCR4 - Chemokine (C-X-C motif) receptor 4, gene

DAMPs - Damage Associated Molecular Pattern Molecules

DGS - General Directorate of Health

DMARDs - Disease-Modifying Antirheumatic Drugs

DNA - Desoxyribonucleic Acid

DOCK10 - Dedicator of Cytokinesis

DKK1 - Dickkopf homolog 1

EP300 - E1A binding protein p300, gene

EPOR - Erythropoietin Receptor, gene

EQ5D - European Quality of Life-5 Dimensions

ERAP1 - Endoplasmic Reticulum Aminopeptidase 1

ESSG - European Spondyloarthropathy Study Group

ESR - Erythrocyte Sedimentation Rate

GP's - General Practitioners

GSK-3β - Glycogen Synthase Kinase 3β

GWAS - Genome Wide Association Studies

HAQ-S - Health Assessment Questionnaire Ankylosing Spondylitis

HBEGF - Heparin-binding EGF-like Growth Factor

HLA - Human Leukocyte Antigen

HRQoL - Health-Related Quality of Life

HSP - Heat Shock Protein

IBD - Inflammatory Bowel Disease

IFNγ - Interferon-gamma

IFNAR1 - Interferon α Receptor 1, gene

IL - Interleukin

IL1R2 - Interleukin 1 Receptor, Type II, gene

IL12B - Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40), gene

IL17R - Interleukin 17 Receptor, gene

IL23R - Interleukin 23 Receptor, gene

ITAM - Immunoreceptor Tyrosine-Based Activation Motif

ITIM - Immunoreceptor Tyrosine-Based Inhibitory Motif

JAK2 - Janus Kinase 2

KREMEN1 - Kringle containing transmembrane protein 1

LBP - Low Back Pain

LD - Linkage Disequilibrium

LIGHT(TNFSF14) - Tumor Necrosis Factor (ligand) Superfamily, Member 14, gene

LMP - Low Molecular Weight Proteosome

LNPEP - Leucyl/cystinyl aminopeptidase, gene

LOX-1 - Lectin-like Oxidized low-density lipoprotein receptor-1, gene

LRP - Low-density lipoprotein Receptor-related Protein

LPS - Lipopolysaccharide

LRR - Leucine-Rich Repeat

MAF - Minor Allele Frequencies

MASES - Maastricht Ankylosing Spondylitis Enthesitis Score

MCP-1 - Monocyte Chemotactic Protein-1, proteasome subunit C2

MHC - Major Histocompatibility Complex

MICL - Myeloid Inhibitory C-type Lectin-like Receptor

MMTV - Mouse Mammary Tumor Virus

MRI - Magnetic Resonance Imaging

MTX - Methotrexate

mSASSS - Modified Stoke Ankylosing Spondylitis Spinal Score

NF - Nuclear factor

NK - Natural Killer

NLRs - NOD-Like Receptors

NLRP - NLR family, Pyrin domain containing gene

NR4A2 - Nuclear Receptor Subfamily 4, group A, member 2 *(NR4A2)*, gene

NSAIDs - Nonsteroidal Anti-Inflammatory Drugs

nsSNP - Nonsynonymous SNP

OR - Odds Ratio

PAMPs - Pathogen-Associated Molecular Patterns

PBMC - Peripheral Blood Mononuclear Cells

PCR - Polymerase Chain Reaction

PCR-rSSOP - Polymerase Chain Reaction-reverse Sequence Specific Oligonucleotide Probe

PCSK6 - Proprotein Convertase Subtilisin/Kexin type 6, gene

PGE2 - Prostaglandin E2

PI3K/AKT - Phosphoinositide-3 kinase/AKT

PLC - Peptide-Loading Complex

Pm - Poor metabolizer

PPP2R1A - Protein Phosphatase 2, Regulatory subunit A, alpha, gene

PRRs - Pattern Recognition Receptors

PsA - Psoriatic arthritis

PTGER4 - Prostaglandin E Receptor 4 (subtype EP4), gene

PTPN1 - Protein Tyrosine Phosphatase, Non-receptor type 1, gene

PYD - Pyrin

QoL - Quality of Life

RA - Rheumatoid Arthritis

RGS1 - Regulator of G-protein signaling 1

RLRs - RIG-I-like Receptors

ROC - Receiver Operating Characteristic

RNA - Ribonucleic Acid

RSV - Respiratory Syncytial Virus

RT-PCR - Reverse Transcription Polymerase Chain Reaction

RUNX3 - Runt-related Transcription Factor 3, gene

SDF-1 - Stromal Cell-Derived Factor-1

SF36 - Short Form (36-Item) Health Survey

SFMC - Synovial Fluid Mononuclear Cells

SNP - Single Nucleotide Polymorphisms

SOC3 - Suppressor of Cytokine Signaling 3

SpA - Seronegative Spondylarthritis

SPARC - Secreted Protein, Acidic, Cysteine-rich (Osteonectin), gene

SPOCK2 -Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican)2, gene

SSZ - Sulphasalazine

STAT3 - **S**ignal Transducer and Activator of Transcription 3 (acute-phase response factor), gene

TAP - Transporter ATP-binding cassette, sub-family B

TAP1 - Transporter 1, ATP-binding cassette, sub-family B

TAP2 - Transporter 2, ATP-binding cassette, sub-family B

TASC - The Australo-Anglo-American Spondyloarthritis Consortium

TBKBP1 - TBK1 Binding Protein 1, gene Transforming Growth Factor- β (TGF β)

TGF β - Transforming Growth Factor- β

TIR - Toll/IL-1 Receptor

TLDA - TaqMan Low Density Array

TLRs - Toll-like Receptors

TNF - Tumor Necrosis Factor

TNFAIP3 - Tumor Necrosis Factor, Alpha-Induced Protein 3, gene

TNFR1 - Tumor Necrosis Factor Receptor 1, gene

TNFRSF1A - Tumor Necrosis Factor Receptor Superfamily, Member 1A, gene

TNFSF8 - Tumor Necrosis Factor (ligand) Superfamily, Member 8, gene

TNFSF15 - Tumor Necrosis Factor (ligand) Superfamily, Member 15, gene

TRADD - Tumor Necrosis Factor Receptor Superfamily, Member 1A - Associated via Death Domain, gene

UPR - Unfolded Protein Response

uSpA - Undifferentiated Seronegative Spondylarthritis

VAS - Visual Analogue Scale

VEGF - Vascular Endothelial Growth Factor

WTCCC - Wellcome Trust Case Control Consortium

WNT - Wingless-type MMTV Integration Site Family

BOOK CHAPTERS AND ARTICLES

BOOK CHAPTERS

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1. INTRODUCTION

Ankylosing spondylitis (AS) is the prototypic disease of a group of arthropathies called seronegative spondyloarthritis (SpA). It represents a group of related conditions that also includes arthritis associated with inflammatory bowel disease (IBD), psoriatic arthritis, arthritis associated with acute anterior uveitis (AAU), reactive arthritis, and undifferentiated SpA (uSpA) [Rudwaleit *et al.,* 2009a; 2010]. Collectively, all these conditions have a common background of inflammatory arthritis (predisposition to axial arthritis involving the spine and sacroiliac joints but also peripheral arthritis), extra-articular inflammation, seronegativity for rheumatoid factor and a strong *HLA-B27* association [Dougados *et al.*,1991; Calin & Taurog, 1998]. Inflammatory back pain is a characteristic symptom; new bone formation with syndesmophytes and ankylosis, are the disease radiographic hallmark. Histopathologically, AS is characterized by the presence of enthesis inflammation [Benjamin & McGonagle, 2001].

The incidence and prevalence rates mirror the prevalence of *HLA-B27* seropositivity [Khan, 1996] which in turn has a strong population variation. In fact, among adults who are positive for the presence of *HLA-B27*, the prevalence of all types of SpA and AS in particular, were estimated at 4.5% and at 1.6%, respectively [Benevolenskaya *et al.*, 1996]. As far as ethnicity is concerned, 8% of Caucasians were found to be *HLA-B27* positive, and to have a prevalence of SpA up to 1.3% and of AS ranging from 0.2% to 0.9% [Braun *et al.*, 1998]. The Portuguese available data comes from a random sample of 1238 people (age > 18 years old) drawn from the Oporto population; in this study a standard questionnaire was used to

ascertain self-reported AS information (albeit one should note this information was not validated by any clinician). In this survey, AS prevalence was estimated at 0.6% [ONDoR, 2003-2005]. In addition, in Terceira Island SPA was found to have a prevalence of 1.6% in people over 50 years old [Bruges-Armas *et al.,* 2002]. SpA and AS are, therefore amongst the most common forms of inflammatory arthritis.

The incidence of AS has already studied in different populations and some discrepancies were found. Similar ratios of 6.4, 6.9, 7.26 and 7.3 per 100,000 people per year were estimated in populations in Czech Republic [Hanova *et al.,* 2010], Finland [Kaipiainen-Seppanenet *et al.,* 1997], Norway [Bakland *et al.,* 2005] and Rochester - USA [Carbone *et al.,* 2005], respectively. However, in other populations, such as Greek, the incidence of AS seems to be significantly lower - 1.5 per 100,000 [Alamanos *et al.,* 2002]. In fact, many challenging factors have made it difficult to determine the exact prevalence and incidence of SpA. Among these, population ethnic heterogeneity, lack of feasibility to apply current criteria, and the transient nature of some SpA symptoms (peripheral arthritis and enthesitis) are to be included [Reveille, 2011]. Nevertheless, in the past few years some general fixed traits in epidemiologic characteristics of AS seem to emerge [Gabriel & Michaud, 2009]. The incidence of AS appears to be relatively stable over time [Kaipiainen-Seppanen *et al.,* 1997; Bakland *et al.,* 2005] or even with a tendency to decline [Bakland *et al.,* 2005].

AS typically affects young people; 80% of the patients develop the first symptoms at an age younger than 30 years, and less than 5% older than 45 years [Feldtkeller *et al.,* 2003]. Men are more often affected than women, with a ratio of roughly 2:1 [Brunner *et al.,* 2002; Feldtkeller *et al.,* 2003]. Some authors have even described higher ratios of 3:1 [Lee *et al.,*

2007] or even of 6-8:1 in some Asian populations [Lee *et al.,* 2002; Zeng *et al.,* 2003; Jung *et al.,* 2010]. The reasons for such gender disparities haven't established yet.

Clinical features include inflammatory back pain, asymmetrical peripheral oligoarthritis (predominantly of the lower limbs), enthesitis, and specific organ involvement such as AAU, psoriasis, and chronic inflammatory bowel disease. Pulmonary, renal, neurological, aortic root involvement and conduction abnormalities are all rare complications of AS [Braun & Sieper, 2007]. However, in accordance to studies performed in European and Asian populations, patients with AS have a higher prevalence of multiple comorbidities than general population [Bremander *et al.,* 2011]. In Taiwanese AS cases, it was documented that there was an increased risk for cardiovascular, neurological, pulmonary, gastrointestinal, endocrine, haematological and mental illnesses. Hypertension (16.4%), peptic ulcers (13.9%) and headaches (10.2%) were the most prevalent findings [Kang *et al.,* 2010]. The increased risk for cardiovascular disease in AS is a consistent finding mentioned in several studies. It is probably multifactorial, being related both to chronic systemic inflammation and to high prevalence of conventional cardiovascular risk factors [Boonen *et al.,* 2002; Mathieu *et al.,* 2010; Bakland *et al.,* 2011]. Moreover, AS also increases mortality by 50% when compared with age- and gender- matched controls [Boonen *et al.,* 2002]. Interestingly, standardised mortality rates seem to be significantly increased only among male patients compared with female patients [Bakland *et al.,* 2011]. Finally, male patients seem to have more structural changes, including bamboo spine, low bone density [Karberg *et al.,* 2005], and an increased rate of fractures [Cooper *et al.,* 1994] which may also contribute to hyperkyphosis [Vosse *et al.,* 2006], in comparison with female patients. Taken together, articular and systemic

involvement, need to be concurrently and comprehensively studied to evaluate the total burden of this rheumatic disease both in terms of individual and societal impact.

In this context, many studies have reported that AS leads to deterioration of physical function and deterioration of health-related quality of life (HRQoL), with repercussions in terms of work disability and in increasing demand of health care services. The best means to understand the burden of the disease in a specific population is to compare it with the general population [Ware, 2000]. Generic measures, such as the Short Form (36 item) Health Survey (SF-36), allow this kind of comparisons, contrary to disease-specific HRQoL measures [Guyatt *et al.,* 1993]. Studies that compared HRQoL of AS patients with general population reported significantly low HRQoL on physical and a less extent on psychosocial domains, when raw scores [Dagfinrud *et al.,* 2004] or age-sex-adjusted scores were considered [Davis *et al.,* 2005; Singh & Strand, 2009]. Thus, it means that physical component may be the main contributor to morbility associated to this condition. Many of the following factors may influence HRQoL: severity and disease duration; response to current therapy; treatment-related adverse events; medical comorbility; socioeconomic factors; and healthcare access. However, as many of these factors may be changed, targeted interventions may also potentially improve function and HRQoL in these patients [Singh & Strand, 2009]. In the other hand, some prognostic factors linked to functional repercussion were identified. The onset of symptoms at a young age yields the worse functional outcomes [Stone *et al.,* 2005]. Additionally, in patients with a disease longstanding of 20 years, functional restrictions are worse in those with a history of physically demanding jobs, more comorbid conditions, and in smokers, than in those with higher levels of education and a family history of AS [Ward, 2005].

These considerations might also partially explain the high levels of work disability and the negative impact on productivity and social costs [Boonen *et al.,* 2002]. In Dutch patients with AS, it has been shown that labour force participation was 11% lower and AS related work disability 15% higher than in general population. In those with a paid job, the mean number of AS related sick leave days per patient was 10 days per year [Boonen *et al.,* 2001]. In terms of health care services demand results from some European studies have reported a range between 1.4 - 4.0 for general consultations and 1.7 - 2.8 for specialist consultations per year incurred by AS patients [Boonen *et al.,* 2003a, 2003b, 2005; Verstappen *et al.,* 2007]. Similar results were obtained from a USA cohort with values higher than 2.1 for outpatient generalist and specialist visits [Ward, 2002]. Based on administrative databases, two previous studies from Canada [Kobelt *et al.,* 2006] and Germany [Zink *et al.,* 2006] reported 16-18 visits to a generalist and 3.7-4.2 visits to a rheumatologist annually. Health care delivery systems, countries, studied cohorts, patient characteristics (including presence of medical comorbidities and disease severity) and the method for ascertaining health care demand may explain these differences. However all studies documented an increase in health care services demand. Many possible reasons can be pointed out in order to explain this increase:

a) Primary care need is associated with symptoms directly related with SpA/AS (back pain, joint pain, enthesitis) and/or presence or severity of other comorbid conditions and/or treatment-related complications;

b) Surgical clinic need is related to SpA consequences (e.g. hip or knee arthroplasty) and/or related to associated comorbidities;

c) Specific rheumatology visits are likely to reflect regular follow-up to rheumatology clinic for treatment of SpA and the monitoring of these therapies;

d) Other specialty clinics, like gastroenterology, nephrology and cardiology, for SpA/AS or therapeutic associated complications.

It is also important to analyse AS impact in terms of costs. Annual total costs averaged US$ 6,720 (in 1999, median US$ 1,495). Indirect costs comprised 73.6% and direct costs comprised 26.4% of total costs, although only 39% of patients contributed to indirect costs [Ward, 2002]. Functional disability was the most important predictor of high total costs. Likelihood of having high (>US$ 10,000) total costs increased by a factor of three with each single-point increase in Health Assessment Questionnaire disability index modified for the SpA (HAQ-S; range 0–3) [Ward, 2002]. In one study conducted in Portugal, a statiscally significant correlation between total cost (supported by the patient) and BASDAI and HAQ was observed [Miranda *et al.*, 2008]. Therefore, interventions that maintain or improve patients' functional ability will likely have the greatest potential to decrease the costs of AS. Likewise, comorbidities might also influence costs. However, further studies are needed to examine the association between increased financial stress upon health care services and comorbidities (both in number and severity); further, it should be also analysed whether a better control of comorbidities reduces health costs in SpA patients. Keen attention should be given to results interpretation of such studies as costs may vary internationally, depending on each country health care system [Boonen *et al*., 2002].

All factors considered together (disease prevalence, likely irreversibility of progressive ankylosis in affected joints, comorbidities, and socioeconomic impact of disease), it is reasonable to suggest that early diagnosis and effective treatment offer the best chances of improving prognosis; this approach stands for the present paradigm shift.

Several studies have shown a delay of more than eight years between the onset of symptoms and diagnosis, with consequent delay in starting therapy [Feldtkeller *et al.*, 2003]. This is a critical period for clinical damage.

The modified New York criteria [van der Linden *et al.*, 1984], requires the presence of radiographic sacroiliitis for diagnosis purposes. This is a major concern because it has been estimated that after the onset of inflammation in SI joints, a period of six to eight years must elapse before sacroiliitis can be detected on plain radiographs [Rudwaleit *et al.*, 2005, 2009b; Bennettet *et al.*, 2008]. Although the proportion of SpA that evolves to AS is unknown, patients with non-radiographic axial SpA have the same high disease activity as patients with established AS in terms of signs and symptoms [Rudwaleit *et al.*, 2009b]. For this reason, some authors have hypothesised that patients with non-radiographic axial SpA and patients with established AS display different stages of a single disease continuum and, therefore, the same disease entity [Rudwaleit *et al.*, 2005]. Assuming this hypothesis to be true, the new ASAS criteria for axial SpA may allow the identification of early stages of disease, when radiographic sacroiliitis is still abstent.

To assess the efficacy of therapeutical agents, BASDAI 50 [Braun *et al.*, 2003] and ASAS improvement criteria [Anderson *et al.*, 2001] were proposed. It is still unclear whether the changes highlighted by these scores are helpful in patient monitoring. The recently introduced ASDAS seems to be a highly discriminatory instrument for assessing disease activity in AS and therapeutic response [Lukas *et al.* 2009; van der Heijde *et al*., 2009; Machado *et al.*, 2011]. ASDAS is unquestionably of potential usefulness in clinical practice, even if it wasn't initially developed as a predictor of response to therapy. As a matter of fact, and in order to

have some insight into response to therapy, some markers were identified as positive therapy responsiveness, namely: younger age, *HLA-B27* carriage, elevation of acute phase reactants (CRP), and marked spinal inflammation (detectable by MRI). Likewise, other factors were identified as negative markers of therapy response: older age, structural damage and poor function may be predictors of poor- or non-responsiveness [Rudwaleit *et al.*, 2004, 2008]. Although all these markers were found to be of interest due to their statistical significance, each patient is a case of its own and therefore new and more discriminative biomarkers are needed for clinical purposes. The need to personalize treatment is further accentuated by economical reasons; the introduction of anti-TNF alpha drugs (infliximab, etanercept, adalimumab, golimumab) and the expectation of new therapies for different targets are extremely expensive and unbearable by public health systems. In such a context, therapy selection will become an issue and new powerful biomarkers are needed to ensure both correct monitoring of disease and correct and meaningful drug selection and use. Identification of relevant markers for diagnosis, prognosis, and treatment response will render an easier diagnosis and also allow the best choice of treatment, based on therapy likelihood to elicit a positive response. This would have the extra advantage of significantly reducing treatment costs by minimizing the use of expensive therapeutic agents in patients unlikely to respond to them.

In recent years, improvements in genotyping and in study design have had a major impact in AS. New findings provide fascinating insights into the pathogenesis of the disease and offer new potential therapeutic targets [Brown *et al.*, 2010; Evans *et al.*, 2011]. Moreover, two recent studies showed that anti-TNF alpha treatment leads to significant alteration of gene expression and in proteomic profiling; the authors' data support the use of systematic gene

expression and proteomic analysis to shed new light on pathogenic pathways in chronic inflammation of AS and eventually in therapeutic response [Visvanathan *et al.*, 2008; Haroon *et al.*, 2010]. Such findings raise expectations that genetic studies can aid in the development of more discriminating diagnostic methods and the current project is a natural extension of all these recent discoveries.

The specific aim of the current study is to characterize the clinical AS pattern in Portugal and to assess genetic predictors of susceptibility to AS. It was hypothesized that genetic factors may predict the susceptibility to the disease. Additionally, genetic factors may also contribute to predict the prognosis and clinical response to different therapies used.

The aims of the present study are:

a) To collect a representative sample of AS Portuguese patients to characterize the disease (epidemiologically and clinically) in Portugal;
b) To validate the instruments used currently in clinical practice - the Bath AS indices - to Portuguese;
c) To create new tools to monitor AS;
d) To contribute to the identification of biomarkers with potential interest for diagnosis (and secondary for clinical response to therapy and prognosis) in AS patients.

The present research project led to the creation of a work group called CORPOREA - **CO**nhecer a **R**ealidade **Por**tuguesa sobre **E**spondilite **A**nquilosante [Portuguese Ankylosing Spondylitis Knowledge Group]. Many different national, Rheumatology and Physiatry Centres, took part in this work Group namely: Centro Hospitalar de Lisboa Ocidental,

Hospital de Egas Moniz EPE, Lisboa; Centro Hospitalar de Lisboa Norte, Hospital de Santa Maria EPE, Lisboa; Instituto Português de Reumatologia, Lisboa; Hospital Militar Principal, Lisboa; Hospital Curry Cabral EPE; Hospital Garcia de Orta EPE, Almada; Centro Hospitalar do Alto Minho, Hospital Conde de Bertiandos EPE, Ponte de Lima; Hospital de São Marcos, Braga; Hospital de Faro EPE, Faro; Centro Hospitalar Baixo Vouga, Hospital Infante D. Pedro EPE, Aveiro; Centro Hospitalar Oeste Norte, Centro Hospitalar das Caldas da Rainha EPE, Caldas da Rainha. To reduce bias related to recruitment, patients from Azores and Madeira were not included in the present study. Several national (Institute of Molecular Medicine, Faculty of Medicine, University of Lisbon) and international (University of Queensland, Brisbane, Australia; Institut Cochin, Department of Immunology and INSERM, Paris, France), partnerships have been established to bring out necessary conditions for completion of all topics of this project.

By involving multiple national centres, a country dimension was granted to the project. During 6 months, a Rheumatology team from Hospital de Egas Moniz went out to interview, assess, and collect biological samples from patients recruited at each enroled centre. A convenient sample of 369 patients was obtained according to the following criteria:

a) **Inclusion criteria-** Patients that fulfill the modified New York diagnostic criteria for AS; Age ≥ 18 years old; Portuguese ancestry until the second generation; expression of agreement under informed consente to participate in this research project.

b) **Exclusion criteria-** Other inflammatory disease besides AS; Age < 18 years old; refusal of participation; unability to give informed consent.

Biological samples from healthy individuals were collected as a control group. Some samples from the National bone marrow donors' database of Centro de Histocompatibilidade do Sul were also randomly selected.

This research project received favorable evaluation of the Centro Hospitalar de Lisboa Ocidental Ethics Committee and from all Hospitals involved.

The present work is organized in three chapters. Each one of them reflects different stages of research:

a) Chapter I, is on clinical nature. It reports the characterization of Portuguese patients with AS. Bath indices validated to Portuguese are disclosed. Finally, normative charts for BASDAI, BASFI, BASMI, and mSASSS for both genders are reported.

b) Chapter II, is based on genetic DNA studies. There, one can find a report of experiments performed to ascertain validity of several candidate genes in the Portuguese population (and to find variants associated with disease susceptibility/severity).

c) Chapter III, is based on genetic RNA studies where microarray technology was used to identify new candidate genes associated with disease susceptibility.

2. SCIENTIFIC RESEARCH

2.1. CLINICAL RESEARCH

2.1.1. Validation of Bath indices in Portuguese

Pimentel-Santos FM, Pinto LT, Santos H, Barcelos A, Cunha I, Branco JC, Ferreira PL. **Portuguese version of the bath indexes for ankylosing spondylitis patients: a cross-cultural adaptation and validation.** *Clin Rheumatol*. 2012 Feb; 31(2): 341-6.

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ORIGINAL ARTICLE

Portuguese version of the bath indexes for ankylosing spondylitis patients: a cross-cultural adaptation and validation

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Abstract The Bath Ankylosing Spondylitis Activity Index (BASDAI), Functional Index (BASFI), Metrology Index (BASMI), and Global Score (BASG) are commonly used to assess patients with ankylosing spondylitis (AS). The aim of this study was to cross-culturally adapt and validate these indexes into the Portuguese language. Seventy-eight patients were included in the study. After forward and backward translations, the questionnaires were administered and tested for internal consistency, test–retest reliability, face validity, content validity, and construct validity. The outcome measures HAQ, EQ-5D, and SF-36 were also implemented. Metrological parameters (BASMI components) and chest expansion were evaluated. Correlation coefficients for test–retest were 0.875, 0.937, 0.831, and 0.961 for BASDAI, BASFI, BASMI, and BASG, respectively. Internal consistency coefficients were between 0.747 and 0.953. The adapted and translated questionnaires demonstrated an acceptable comprehensibility by a panel of patients, and face validity was assured by the cognitive debriefing performed. Content validity was assured by comparing the scores obtained by the questionnaires when age and gender, age of symptoms onset, and disease duration were considered. Construct validity was assured by significant correlations established between the Bath scores and generic health status HAQ, EQ-5D and SF-36, morning stiffness duration, chest expansion, and physician disease activity assessment. The Portuguese version of the BASDAI, BASFI, BASG, and BASMI showed adequate reliability and validity in patients with AS. The measurement properties were comparable to versions in other languages, indicating that the indexes can be used for evaluation of Portuguese-speaking AS patients.

Keywords Ankylosing spondylitis · Bath indexes · Validation

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Introduction

The comprehensive and brief international classification of functioning and health core sets for ankylosing spondylitis (AS) were recently proposed by the Assessment in Ankylosing Spondylitis International Working Group. These included spinal stiffness, fatigue, pain, patient's

global assessment, physical function, and spinal mobility [1]. To measure some of the considered domains, several indexes were recommended. The Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) [2] and the Bath Ankylosing Spondylitis Global Score (BASG) [3] measure disease activity while the Bath Ankylosing Spondylitis Functional Index (BASFI) [4] measures functioning and the Bath Ankylosing Spondylitis Metrologic Index (BASMI) [5] measures mobility. Some of the specific items of BASDAI may also be used to assess fatigue or stiffness. All these indexes were developed at the Royal National Hospital for Rheumatic Diseases in Bath, UK, and have been demonstrated as reliable, valid, and sensitive to change [2–5]. The worldwide distribution of AS and the importance of these indexes regarding disease monitoring in clinical practice create the need of homogenous assessment of activity, functioning, and mobility. Therefore, these indexes have been cross-culturally adapted for use in numerous languages. The aim of this study was to translate, culturally adapt, and validate the original English versions into the Portuguese language.

Materials and methods

The adaptation of these indexes involved three main steps: translation, field testing (content validity), and validation study. Ethics committee approval was not required for this proposal.

Indexes

The BASDAI index consists of six horizontal 10-cm visual analog scales (VAS), scored 0–10, to evaluate severity of fatigue, spinal pain, peripheral joint pain, localized tenderness, and morning stiffness (both severity and duration). A score of 0 means "no symptoms," and a score of 10 means "very severe symptoms." To determine a final index score, an equal weight is attributed to each symptom, but, before averaging all the answers, a mean of the two scores relating to morning stiffness is computed [2].

The BASFI index consists of ten questions, also measured by a 0–10-cm VAS, to evaluate the respondents' perception of their functional ability (first eight questions) and how well they are able to function in everyday life (last two questions). A score of 0 means "easy" and a score of 10 means "impossible" at the opposite anchor. The mean score (range, 0–10) is calculated across all items [3].

The purpose of BASG is to make a global assessment of the well-being of a person with AS over a given period of time: last week and the last 6 months. The score ranges from 0 ("none") to 10 ("very severe effect"), and the mean of the two scales gives the final BASG score between 0 and 10 [4].

At last, the metrology index BASMI is a composite score involving five measurements: cervical rotation, wall–tragus distance, lumbar lateral flexion, modified Schöber test, and maximum intermalleolar distance. Each of these parameters is scored between 0 and 2, depending on the disease involvement (0: mild, 1: moderate, 2: severe disease involvement). The sum of all measurement scores is divided by 5, giving the BASMI value with a final score ranging from 0 to 2 [5].

Translation

The translation process followed scientifically agreed guidelines [6]. All the Bath Indexes were used as stating point with permission from the corresponding authors. After their agreement, the indexes were translated into Portuguese, separately, by a bilingual panel familiar with the medical aspects of AS and by one Portuguese professional translator. The two resultant translations were semantically, idiomatically, and conceptually examined and converted on a first preliminary version by a first-consensus panel composed by two rheumatologists (JCB, FPS) aware of the purpose of the study and by another researcher (PLF) familiar with validation processes.

This consensus version was separately back-translated by two English professional translators unaware of the original version. Both English versions were compared to obtain a second Portuguese preliminary version, and some modifications were made to reach an agreement.

The last step was a clinical review performed by one of the rheumatologists (JCB) who looked at the more technical aspects of the questionnaires, taking into account the best way to communicate with patients. For each item of the questionnaires, this expert faced both the original version in English and the proposed Portuguese translation. This reviewer was then asked to accept the Portuguese version or to propose another version for further tests with patients.

Field test interviews

To complete the process of achieving semantic equivalences of the original indexes, and to assure that all items of the questionnaires were comprehensible, the latest Portuguese consensus version passed through field test interviews. Six males and three females with AS attending the Rheumatology Clinic of Egas Moniz Hospital in Lisbon were interviewed to test face validity and content validity of the questionnaires. All of the patients fulfilled the modified New York criteria for AS [7]. The mean age was 41.1 years (median of 40 and ranging from 28 to 56 years). The participants completed the questionnaires and were subsequently interviewed by one researcher (PLF). Respondents were invited to identify the problems in answering the

questions and to comment on the comprehensiveness and the relevance of the items and scales used in the indexes. The field test interviews were conducted in two different occasions: During the first moment, some suggestions were made, and at the second moment, the participants received two different examples of the same questionnaire, improved according to previous suggestions. Respondents were asked to state which question wording and which scales they would prefer and the reasons for their preferences.

Validation study

The sample involved 78 AS patients from three Portuguese Rheumatology Departments (CHLO-Egas Moniz Hospital, Instituto Português de Reumatologia and Infante Dom Pedro-Aveiro Hospital), over 1 year (2009). The diagnosis was confirmed by one rheumatologist from each department according to the modified New York criteria, 1984 [7], for AS. The survey included socio-demographic data, BASDAI, BASFI, BAS-G, and the Portuguese versions of The Health Assessment Questionnaire (HAQ) [8], Short-Form Health Survey 36 (SF-36) [9, 10], and EuroQoL-5D (EQ-5D) (http://www.euroqol.org/eq-5d/eq-5d-products/eq-5d-3l-translations.html). The physician disease activity was evaluated through a 0–10-cm horizontal VAS, the BASMI components (cervical rotation, wall–tragus distance, lumbar lateral flexion, modified Schöber test, maximum intermalleolar distance), and additionally, the chest expansion.

Statistical analysis

Reliability Internal consistency was assessed by Cronbach's alpha coefficients. An alpha score between 0.70 and 0.95 was considered as acceptable reliability [11]. Reproducibility over 1 week without changes in treatment was also evaluated using Spearman's rank correlation coefficients and interclass correlation coefficients. Correlation coefficients of 0.85 or above were required to indicate acceptable levels of measurement error, but, if greater than or equal to 0.70, is considered as a positive rating [11].

Validity The Spearman's correlation coefficients were performed and interpreted as follows: excellent relationship, greater than 0.90; good, between 0.90 and 0.71; fair, between 0.70 and 0.51; weak, between 0.50 and 0.31; and little or none, lower than 0.30 [12]. A p value was significant if less than 0.05. Criterion validity was assessed by relating BASDAI, BASFI, BASG, and BASMI scores with other scores such as HAQ, SF-36, EQ-5D, morning stiffness duration, chest expansion, and physician disease activity assessment.

We hypothesized that BASDAI would have a high correlation with general health thermometer (EQ-5D VAS), EQ-5D pain/discomfort sub-scale, bodily pain sub-dimension and physical summary of SF-36, physician disease activity assessment (VAS), and, morning stiffness duration. BASFI and BASMI would correlate with HAQ; Mobility, Self-care, and Usual activities EQ-5D sub-scales; Physical function, Role physical sub-dimensions, and physical summary of SF-36; and chest expansion. BASG would correlate with HAQ, EQ-5D index, EQ-5D VAS, and SF-36 sub-dimensions, in particular, with general health.

Floor/ceiling effects Floor/ceiling effect was considered present if more than 15% of the patients give the lowest/highest possible score.

Statistical analyses were performed using the Statistical Package for the Social Sciences, SPSS version 18.

Results

Field testing

The second Portuguese version of the indexes was very well-accepted by patients. Filling in the BASDAI, BASFI, and BASG questionnaires took respectively 1.8, 3.0, and 1.2 min. However, some patients suggested the introduction of an example on how to complete the VAS. In addition, more than 50% of patients understood intuitively the VAS as a continuous scale. Regarding the clinical review, only minor errors were identified. The corrections were made immediately. On the other hand, the panel of rheumatologists considered to be necessary to change the words of item 6 of the BASDAI for clarity. It was consensual that all indexes needed a Portuguese title in addition to the acronyms BASDAI, BASFI, BASG, or BASMI.

Validation study

A total of 78 AS patients (69.2% were male) with a mean age of 44.8 years were included for the reliability, validity, and floor/ceiling effects. Table 1 shows a description of the demographic and clinical characteristics of the involved group.

Reliability

The Cronbach's alpha coefficients for all the studied indexes range from 0.747–0.953, showing acceptable internal consistency. In addition, the reproducibility (test–retest) was evaluated by applying the same index with 1-week interval. Spearman's rank correlation coefficients were 0.875, 0.937, 0.831, and 0.961 for BASDAI, BASFI,

Table 1 Demographic and clinical characteristics of AS patients in baseline (n=78)

Characteristics	n	Mean (SD; min–max)
Gender: female	24 (30.8%)	NA
Age, years	78	44.8 (11.9; 23–70)
Age of symptom onset	78	28.1(12.6; 6–66)
Disease duration, years	78	17.6 (13.0; 1–50)
Morning stiffness duration	77	31.88 (37.15; 0–180)
BASDAI	77	3.14 (2.01; 0.12–8.05)
BASFI	78	3.63(2.65; 0–9.85)
BASG	77	4.72 (2.55; 0.10–10)
BASMI	77	3.45 (2.41; 0–9)
HAQ	67	1.31 (0.89; 0–3)
EQ-5D	77	0.59 (0.31; −0.29–1)
SF-36—physical function	78	61.74 (25.44; 10–100)
SF-36—role physical	78	61.54 (28.89; 0–100)
SF-36—bodily pain	78	48.78 (23.31; 0–100)
SF-36—general health	78	41.81 (18.64; 0–92)
SF-36—vitality	78	48.65 (25.82; 0–100)
SF-36—social functioning	78	71.63 (26.01; 0–100)
SF-36—role emotional	78	70.41 (27.05; 8.33–100)
SF-36—mental health	78	66.92 (22.84; 4–100)
Morning stiffness	57	42.54 (37.58; 5–180)
Chest expansion	77	3.63 (1.89; 0–7.50)
Physician disease activity assessment	76	2.73 (2.13; 0–7.70)

BASDAI Bath Ankylosing Spondylitis Activity Index, *BASFI* Bath Ankylosing Spondylitis Functional Index, *BASG* Bath Ankylosing Spondylitis Global Score, *BASMI* Bath Ankylosing Spondylitis Metrology Index, *HAQ* Health Assessment Questionnaire, *EQ-5D* EuroQoL-5D, *SF-36* Short-Form Health Survey 36, *SD* standard deviation, not applicable

BASG, and BASMI, respectively. All indexes but one (BASG, whose value is marginal) achieved acceptable correlations. The results, presented in Table 2, indicate that the indexes can be considered as one-dimensional.

Validity

To assess construct validity, several assumptions were made trying to correlate the studied indexes with others parameters, as described in the "Materials and methods" section. Table 3 shows the Spearman's correlation coefficients obtained and its significance. As expected the BASDAI Index showed good correlation with HAQ, SF-36 bodily pain sub-dimension and SF-36 Physical summary and fair correlations with general health thermometer (EQ-5D VAS, EQ-5D pain/discomfort sub-scale, and physician disease activity assessment). Interestingly, good or fair correlations were obtained with several functional (SF-36 Physical function, role physical and vitality sub-dimensions, and EQ-5D self-care sub-scale) and mental/social dimensions (EQ-5D anxiety/depression sub-scale, SF-36 mental health, role emotional, social functioning dimensions, and SF-36 mental summary).

BASFI showed excellent correlation with HAQ and good correlations with EQ-5D total index and self-care sub-scale, SF-36 Physical summary, and with SF-36 physical function and role physical sub-dimensions. Fair correlations were established not only with mobility and usual activities EQ-5D sub-scales and physician disease activity assessment as hypothesized, but also with pain (pain/discomfort EQ-5D sub-scale and bodily pain sub-dimension of SF-36) and mental/social aspects (anxiety/depression EQ-5D, role emotional, general health, vitality, social functioning dimensions of SF-36).

Table 2 Reliability tests

Measure	Internal consistency		Intra-class correlation coefficient confidence interval		Test–retest Spearman's correlation	
	n	Cronbach's $\acute{\alpha}$	Lower bound	Upper bound	N	Correlation
BASDAI	77	0.919	0.887	0.944	51	0.875
BASFI	78	0.953	0.935	0.967	51	0.937
BASG	77	0.747	0.445	0.885	41	0.831
BASMI	77	0.785	0.698	0.852	41	0.961

Table 3 Relationship between bath scales and other outcome measures

	BASDAI	BASFI	BASMI	BASG
HAQ-global	0.900**	0.926**	0.328	0.850**
EQ-5D—mobility	0.426**	0.543**	0.241*	0.458*
EQ-5D—self-care	0.549**	0.724**	0.287*	0.609**
EQ-5D—usual activities	0.496**	0.521**	0.210	0.700**
EQ-5D—pain/discomfort	0.580**	0.635**	0.290*	0.580**
EQ-5D—anxiety/depression	0.624**	0.518**	0.148	0.430*
EQ-5D—VAS	−0.673**	−0.576**	−0.338**	−0.868**
EQ-5D—index	−0.691	−0.738	−0.319	−0.701
SF-36—physical function	−0.735**	−0.813**	−0.330**	−0.797**
SF-36—role physical	−0.768**	−0.675**	−0.314**	−0.720**
SF-36—bodily pain	−0.707**	−0.579**	−0.220	−0.612**
SF-36—general health	−0.580**	−0.590**	−0.311**	−0.634**
SF-36—vitality	−0.724**	−0.565**	−0.193	−0.570**
SF-36—social functioning	−0.657**	−0.538**	−0.143	−0.639**
SF-36—role emotional	−0.674**	−0.603**	−0.370**	−0.730**
SF-36—mental health	−0.657**	−0.502**	−0.227*	−0.417*
SF-36—physical summary	−0.771**	−0.764**	−0.322**	−0.779**
SF-36—mental summary	−0.624**	−0.428**	−0.212	−0.467**
Morning stiffness duration	0.479**	0.517**	0.303*	0.542*
Chest expansion	−0.301*	−0.284*	−0.533**	−0.259
Physician disease activity assessment	0.619**	0.601**	0.310*	0.713**

Values in the table represent correlation coefficients

*p<0.05, **p<0.001

On other hand, BASMI showed no correlation with any of the studied variables except a fair correlation with chest expansion.

Finally, BASG showed good/fair correlations, as expected, with HAQ, EQ-5D VAS, EQ-5D total index, and general health in the SF-36 dimension. Once again, several aspects may influence BASG evaluation as pain (EQ-5D pain/discomfort and SF-36 bodily pain sub-dimension) or mental/social repercussion of the disease (role emotional, social functioning, vitality dimensions of SF-36). In all these parameters, good/fair correlations were established. Interestingly, BASG showed a good correlation with physician disease activity assessment.

Floor/ceiling effects

Any of the studied indexes showed floor/ceiling effects. In fact, none of the patients have the lowest or the highest score in BASDAI, the lowest score in BASG, or the highest score in BASFI or BASMI. The proportion of patients with the highest score in BASG was 1.3%, and the lowest score in BASFI or BASMI were 2.6% and 10.4%, respectively.

Discussion

BASDAI, BASFI, BASG, and BASMI are very important tools to assess the impact of AS in terms of clinical practice or for clinical research purposes. Some of the originals English versions have been validated in different languages [13–19] including that for Brazilian–Portuguese speakers [20]. Despite its importance, these indexes were not validated into the Portuguese language. This study aimed to assess the validity, reliability, and comprehensibility of the cross-culturally adapted Portuguese versions of the BASDAI, BASFI, BASG, and BASMI. As commented, the Portuguese adapted versions of these indexes produced in this study maintained all the properties of the original English-language versions of the instruments. In fact, the resulting Portuguese measures showed good psychometric properties, with acceptable test–retest reliability and satisfactory face, content, and construct validity. Thus, disease activity, functioning, and well-being in Portuguese-speaking patients with AS may be adequately evaluated with these versions of the original instruments.

We have decided not to change the original 0–10 cm VAS scales to numeric rating scales, as proposed by other authors [13, 15, 17]. Beyond some methodological reasons, we want to culturally adapt a measure to another language and not to create a new one. The major disadvantage of the VAS, reported by some authors, is that, although it is simple in conception, responders (in particular, among the elderly and illiterates) often find it difficult to interpret. Curiously, in our study, the responders did not feel any difficulties in using the VAS. On the other hand, the major advantage of a VAS is that it is impossible for the subjects to encode their past answers, if the test is repeated over time.

The reliability of these fouth versions in the 1 week test–retest showed acceptable/positive intraclass correlation coefficients (0.831–0.961). A week's time frame was selected to minimize any change in the patient's clinical condition that would affect reliability and to minimize potencial recall bias. The adminstration of three different instruments also contribute to make it unlikely that they would remember specific items on all of them. The reported reliability of the BASDAI, BASFI, BASG 1 week, BASG 6 months, and BASMI were 0.93. 0.89, 0.84, 0.93, and 0.92, respectively, in the original papers [2–5].

In agreement with the study done to adapt the Bath measures on disease activity and function into Danish and Arabic [17, 18], the major obstacle regarding validation of such scales is the lack of gold standard for disease activity or function in AS. This validation did not include measurement of response to treatment, and therefore, the sensitivity to change was not determined. To assess the validity of the Portuguese questionnaires in this study, patients' responses were compared with more than one external measure, as mentioned. The validation therefore illustrates the properties of the measures only. Therefore, it cannot be estimated whether the measures objectively record the pain, disability, or function of the individual. Thus, as put in evidence, several emotional or social aspects would probably influence the scoring on the scales, and the existence of correlations between these indexes is well-known. In this context, it is curious to put in evidence on the relevance of anxiety/depression EQ-5D sub-scale and all the mental health SF36 domains in BASDAI scores. In addition, the functional repercussion seems to exert an influence on how patients assess disease activity. On the other hand, the well-being assessed through BASG and the perception of functional ability assessed through BASFI are both influenced by pain and, again, by mental/social repercussion of the disease. In contrast, BASMI, as an objective measure performed by clinicians, is not affected by these parameters. The only correlation was established with chest expansion, another objective measure.

In conclusion, results of this work revealed that the Portuguese version of the questionnaires maintained all the properties of the original English-language forms of the instruments. The Portuguese questionnaires were found to be valid, reliable, and comprehensible; hence, these instruments can be applied in clinical practice and for researches purposes.

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Disclosures None

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2.1.2. Clinical and epidemiological characterization of Ankylosing Spondylitis in Portugal

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ORIGINAL ARTICLE

Spectrum of ankylosing spondylitis in Portugal. Development of BASDAI, BASFI, BASMI and mSASSS reference centile charts

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Abstract The availability of population-specific normative data regarding disease severity measures is essential for patient assessment. The goals of the current study were to characterize the pattern of ankylosing spondylitis (AS) in Portuguese patients and to develop reference centile charts for BASDAI, BASFI, BASMI and mSASSS, the most widely used assessment tools in AS. AS cases were recruited from hospital outpatient clinics, with AS defined according to the modified New York criteria. Demographic and clinical data were recorded. All radiographs were evaluated by two independent experienced readers. Centile charts for BASDAI, BASFI, BASMI and mSASSS were constructed for both genders, using generalized linear models and regression models with duration of disease as independent variable. A total of 369 patients (62.3% male, mean±(SD) age 45.4±13.2 years, mean±(SD) disease duration 11.4±10.5 years, 70.7% B27-positive) were included. Family history of AS in a first-degree relative was reported in 17.6% of the cases. Regarding clinical disease pattern, at the time of assessment 42.3% had axial disease, 2.4% peripheral disease, 40.9% mixed disease and 7.1% isolated enthesopatic disease. Anterior uveitis

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(33.6%) was the most common extra-articular manifestation. The centile charts suggest that females reported greater disease activity and more functional impairment than males but had lower BASMI and mSASSS scores. Data collected through this study provided a demographic and clinical profile of patients with AS in Portugal. The development of centile charts constitutes a useful tool to assess the change of disease pattern over time and in response to therapeutic interventions.

Keywords Ankylosing spondylitis · Charts · Epidemiology

Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disorder characterized by inflammation in the spine and sacroiliac joints leading to progressive joint ankylosis. Peripheral joints and entheses are frequently involved, and inflammation may involve extra-articular sites such as the uvea, aorta, heart, lungs and kidneys. AS is a common cause of inflammatory arthritis worldwide, with a prevalence of 0.2–0.9% in white European populations [1], but its aetiology is still incompletely understood. AS typically affects young people, with symptoms onset usually occurring in the early 20s, leading to progressive deterioration of physical function and quality of life. Work disability is higher in AS patients than expected in the general population, with a remarkable impact on productivity and social costs [2], and there is some evidence of increased mortality [3].

Assessment of disease activity and severity in AS is of clinical utility in determining current disease status, prognosis and the effects of therapeutic interventions. The BASDAI [4] and BASFI [5] self-administered patient questionnaires are the most widely used tools for the assessment of AS activity and functional status. Metrology is also widely used, providing an objective measure of the effects of the disease on joint mobility; the BASMI [6] is generally the scoring tool for AS metrology. Finally, the mSASSS [7] scores radiographic features of AS and is now considered the preferred method for measurement of damage progression in patients with AS. Reference centile charts have been developed for AS British patients for BASDAI, BASFI and BASMI [8], but no published charts are available from any other population. Furthermore, reference centile charts have never been developed for mSASSS in any studied population.

Well characterised differences between ethnic groups regarding clinical manifestations of some other major inflammatory conditions, such as systemic lupus erythematosus [9], have been described, but there is little data about inter-ethnic differences in AS patients. Characterising differences between ethnic groups is an important process, potentially guiding research into determinants of disease manifestations and severity and being of obvious relevance for the clinical management in the different ethnic groups concerned.

The aim of the present study was to characterise AS clinical manifestations in Portuguese AS patients and to construct reference centile charts for BASDAI, BASFI, BASMI and mSASSS in our population.

Materials and methods

We collected cross-sectional data on Portuguese AS patients, between April 2007 and April 2008. Cases were recruited from patients attending outpatient clinics in nine out of 14 hospitals, which are the main rheumatology referral centres in their regions. The hospitals involved represent two thirds of all rheumatology centers operating in the Portuguese Hospital National Health System (NHS). Nationwide, the hospitals participating in our study

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accounted for approximately 75.6% of all rheumatology visits in 2007 in Portugal. These centres were located in seven different cities, including urban and rural zones, and represent a broad socio-demographic spectrum of the population treated by the NHS. The patients were selected consecutively in each center in the 2 months prior to the visit of the interviewers. Four previously trained rheumatologists were responsible for the data collection and physical examination of all patients enrolled in each involved centre. This procedure facilitated a consistent data collection and ensured a high response rate. Written informed consent was obtained from all study participants, and the study was approved by the Ethics Committee of the University Hospital Centro Hospitalar de Lisboa Ocidental, Hospital de Egas Moniz and by the Ethics Board of the involved centres.

Data collection

All patients were selected according to the inclusion criteria: (a) AS defined by the modified New York criteria [10], (b) age above 18 years old, and to the exclusion criteria: (a) other types of spondylarthritis distinct from AS. Patients completed a questionnaire concerning demographic characteristics, disease features and the BASDAI and BASFI questionnaires. Age at disease onset was defined as the age at symptom onset, and disease duration was defined as the period of time (years) after symptoms onset. For the evaluation of the disease status, the following anthropometrical measures included in the Bath Ankylosing Spondylitis Metrological Index (BASMI) were used and performed: tragus-to-wall distance, modified Schober's test, lateral flexion of lumbar spine, cervical rotation and intermalleolar distance. All patients had pelvic x-rays performed confirming the presence of at least grade 2 bilateral, or unilateral grade 3, sacroiliitis. The modified Stoke Ankylosing Spondylitis Severity Score (mSASSS) [7] was determined when cervical and lumbar x-rays were available (n=213); all radiographs were scored independently by two of us (FS, AFM). Where there was discordance between the scores, they were re-evaluated together by both reviewers, and a consensus score was obtained. Laboratory tests including ESR and HLA-B27 status, determined by sequence-specific single-stranded oligonucleotide probes (SSOP), were evaluated in all patients. Current treatments including non-steroidal anti-inflammatory drugs, glucocorticoids, disease-modifying anti-rheumatic drugs and biological therapies were recorded.

Statistical analysis

Descriptive statistics are presented as mean±standard deviation when referring to quantitative variables and in absolute frequencies and percentages when referring to qualitative variables. The non-parametric Mann–Whitney–Wilcoxon test was used to compare groups in the presence of skewed data.

Centile charts, showing the 5th, 10th, 25th, 50th, 75th, 90th and 95th centiles, were estimated for males and females separately using alternative models for mean and standard deviation for each Bath AS indexes and mSASSS.

The centile curves were calculated using the following equation:

$$\text{centile} = \text{mean} + K \times \text{SD}$$

where K is the corresponding centile of the standard Gaussian distribution.

The mean values were estimated by the fitting Generalized Linear Models (GLM) for each Bath AS indexes and mSASSS with disease duration as covariate. We tried to fit the best model according to the data structure. We used the Gamma, Poisson and Gaussian families and log and identity function as link functions. The quality of adjustment was assessed by the residuals analysis.

The standard deviation (SD) was estimated by linear regression using the absolute value of the residuals from the GLM regression multiplied by $\sqrt{\pi/2} = 1.253$ as the dependent variable and the disease duration as the independent variable.

Socio-demographic and clinical determinants of each Bath AS index were investigated by multivariate regression analysis using GLM. Alternative models specification was assessed with the Akaike information criterion (AIC), the Bayesian information criterion (BIC), deviance and Ramsey reset test. Normality tests and residual analyses were done to check the assumptions of models.

Coefficients with p-value <0.05 were considered significant. The statistical analysis was performed with Stata SE 10 software.

Results

Eleven outpatient clinics from seven different cities were involved from mainland Portugal, representing a broad spectrum of the population treated in the Hospitalar Portuguese Health System. A total of 369 patients were included (62.3% men and 37.1% women), with a mean age of 45.4±13.2 years (range 20–79 years). Table 1 shows the average values of disease duration, age of symptom onset, age of diagnosis and diagnosis delay for the whole group and by sex. The mean disease duration was 11.4±10.5 years (range 0–46 years), the mean age of disease onset was 26.5± 10.8 years and the mean age at diagnosis was 34.1± 12.4 years. The mean delay between onset of symptoms

Table 1 Characteristics of the overall cohort and analysis by gender

	Total	Male	Female	*p*-value*
Gender (*n*;(%))	369 (100%)	232 (62.8%)	(137) 37.1%	–
Age (years)	45.4±13.2	45.7±13.5	44.9±13.9	0.52
Age at onset (years)	26.5±10.8	25.8±10.8	27.5±10.8	0.185
Age at diagnosis (years)	34.1±13.4	33.0±12.3	35.8±12.4	0.040
Time of evolution (years)	18.9±12.7	19.8±12.6	17.6±13.0	0.068
Disease duration (years)	11.4±10.5	12.6±11.0	9.5±9.3	0.022
Diagnosis delay (years)	7.6±9.0	7.1±9.0	8.3±9.0	0.081
Patient global assessment(cm)	4.7±2.4	4.3±2.5	4.8±2.3	0.072
Physician global assessment (cm)	2.6±1.9	2.5±1.9	2.6±1.8	0.36
				<0.001[a]
BASDAI	4.2±2.3	3.7±2.2	4.9±2.3	<0.001
BASFI	4.1±2.7	3.8±2.6	4.5±2.7	0.010
BASMI	4.0±2.5	4.3±2.6	3.5±2.2	0.006
mSASSS	20.9±23.1	27.4±24.6	9.8±14.7	<0.001
ESR (mm/h)	21.7±17.7	20.1±17.4	24.3±18.2	0.012
HLA-B27 positivity (*n* positive: *n* total, (%))	290:360 (80.5%)	183:220 (83.2%)	107:140 (76.4%)	–

Mean ± standard deviation

*Mann–Whitney–Wilcoxon test

[a]*t*-test comparison between patient and medical evaluation

and diagnosis was 7.6±9.0 years and was less than 1 year in 51 cases (13.8%) and longer than 10 years in 86 cases (23.3%). There was no difference in the age of symptom onset between males and females (25.8 vs 27.5, p=0.185), although males were diagnosed at a slightly earlier age (33.0 vs 35.8 years, p=0.040). Juvenile onset (age <16 years) was reported in 39 (10.6%) of cases, whereas late onset (age > 40 years) was reported in 37 (10%). Family history of AS, in a first-degree relative, was reported in 65 of 369 (17.6%) patients.

Lower back pain (42.3%) was the most common initial manifestation. At the time of assessment for this study, 49.9% had axial disease, 2.4% peripheral disease, 40.9% mixed disease and 7.1% isolated enthesopatic disease. Extra-articular manifestations were experienced by 35.2% of the patients, with anterior uveitis (33.6%) being the most common feature. Other associated extra-articular manifestations were less frequent: psoriasis (6.2%), coexistent inflammatory bowel disease (2.4%), pulmonary disease (1.4%), cardiac disease (1.1%) and renal disease (0.3%).

Table 1 summarizes also data about disease activity and its functional and structural repercussion. The patient and physician global assessment (4.7 vs 2.6, p<0.001) differed significantly, with physicians scoring disease severity lower than patients. The mean value for BASDAI was 4.2±2.3, for BASFI 4.1±2.7, for BASMI 4.0±2.5 and for mSASSS 20.9±23.1 (obtained from a sub-group of 213 patients that had a complete radiological evaluation, including total cervical and lumbar x-rays). Compared with men, the mean BASDAI in women was 1.2 points higher (4.9 vs 3.7, p< 0.001) and the mean BASFI was 0.7 points higher (p= 0.010), but the mean BASMI was 0.8 points lower (p= 0.006) and the mean mSASSS was 17.6 points lower (p< 0.001). Comparing the 51 (13.8%) of patients diagnosed with less than 1 year of diagnosis delay with the 86 (23.3%) diagnosed with more than 10 years of diagnosis delay, no significant differences were noted in disease duration adjusted scores (data not shown).

In the overall cohort the mean ESR was 21.7±17.7 mm/h. Regarding HLA B27, 290 of 360 (80.5%) were positive.

The spectrum of the different treatments that patients received at the time of inclusion visit were also depicted. The majority of AS cases were taking NSAIDs (79.1%), and corticosteroids were being used by 17.6% of cases. DMARD were being used by 48.5% of the patients; of these cases 40.8% had only axial involvement. Sulphasalazine in monotherapy was taken by 30.9% of the patients, methotrexate in monotherapy by 8.4% and 6% were taking both. TNF-blockade was being used by 22% of the patients of whom 40.7% had only axial disease. Multiple linear regression analysis revealed a statistically significant higher [1.5 (95% CI: 0.2; 2.8)] BASMI score in patients taking anti-TNF-α therapy (model not shown) as compared to the remaining patients.

Centile charts were developed from a final study population comprising 326 patients for BASDAI, 323 patients for BASFI, 301 for BASMI and 206 for mSASSS. Centile charts were constructed for both genders, showing the 5th, 10th, 25th, 50th, 75th, 90th and 95th centiles (Fig. 1). Our cross-sectional study shows that BASFI,

Fig. 1 BASDAI, BASFI, BASMI and mSASSS reference centile charts for both genders, showing the 5th, 10th, 25th, 50th, 75th, 90th and 95th centiles

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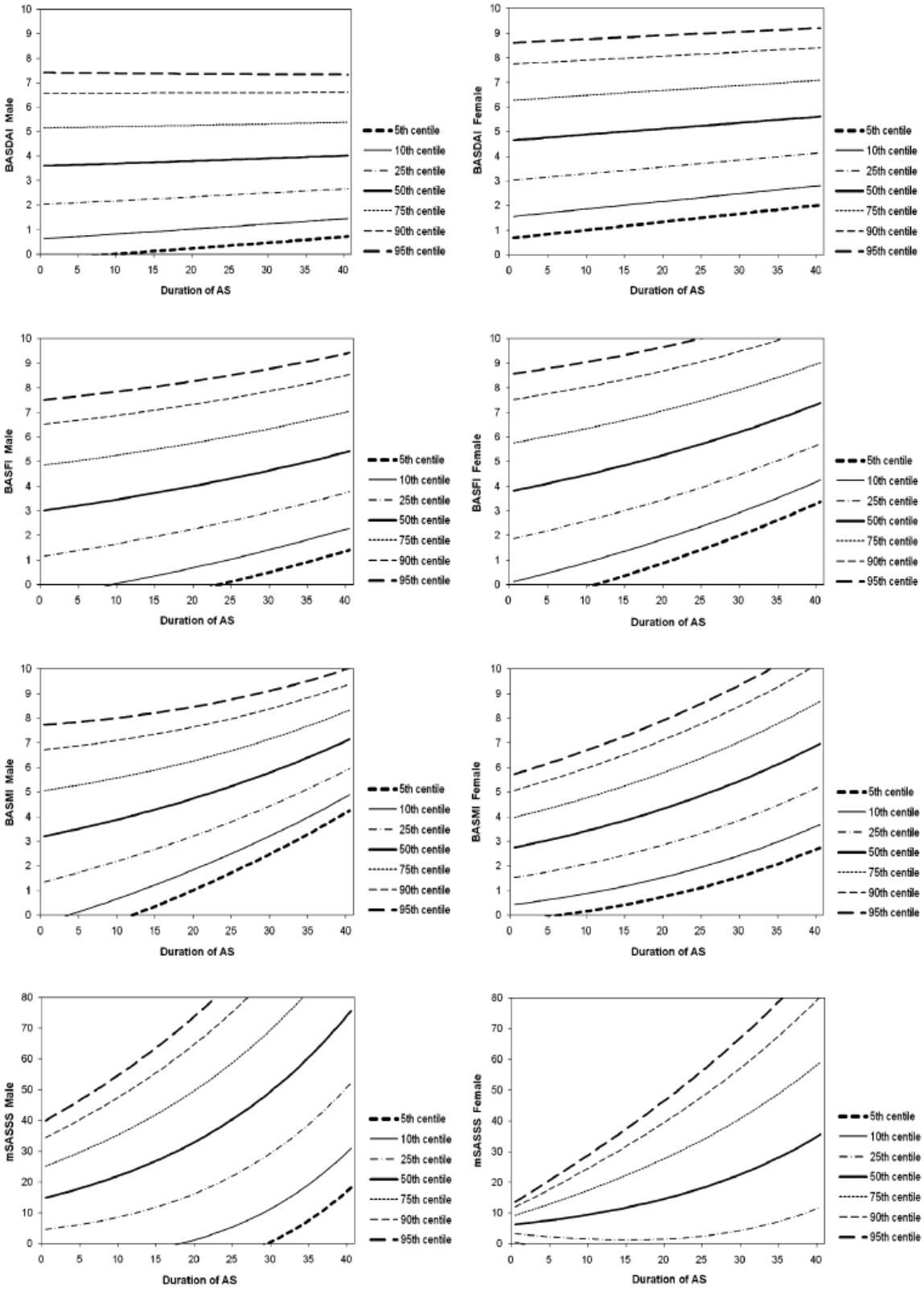

BASMI and mSASSS scores continue to increase as a function of disease duration, even after >20 years, in both males and females. By contrast, disease activity assessed by BASDAI did not change over time.

Comparison of AS between genders

As in other populations, in this study AS affected more frequently males than females. The average age, age at symptom onset and the diagnostic delay were similar in both genders. Females reported greater disease activity (BASDAI) and functional impairment (BASFI) but had better metrology (BASMI) and better radiological evaluation (mSASSS). These differences were valid for the results as a whole but also when different periods of disease duration were evaluated.

Discussion

We report here the first characterization of a Portuguese cohort of AS patients. This study describes the socio-demographic, clinical, radiological and biological profile of AS in our country. Furthermore, we have developed reference centile charts for indices of disease activity, function, metrology and mSASSS in our population. This study involved patients recruited from hospital outpatient clinics, a patient population likely to have more severe disease than the overall AS population. Therefore, our centile charts will need to be assessed in a community-recruited AS case cohort before they can be used in non-hospital based settings.

This study confirms that AS in Portugal have similar characteristics to the disease pattern described in other European populations. There is a striking male predominance (62.3% males vs 37.1% females), and the age at symptom onset (26.5±10.8 years) and the frequency of extra-articular features were similar to those reported in previous Portuguese studies [11, 12] or in other studies of white European ethnic groups [13, 14].

Diagnostic delay in this cohort was similar to that reported in other developed countries (7.6±9.0 years) [13–19]. An early diagnosis (less than 1 year after starting symptoms) was established in only 13.8% of the cases. There are many potential explanations for this, but one of the contributors is likely to be the low sensitivity of the New York classification criteria when used in the clinical practice. It will be interesting to observe whether diagnostic delay is reduced with the daily use of the Assessment of SpondyloArthritis international Society (ASAS) undifferentiated spondylarthritis criteria [20]. Interestingly, no statistical difference was found between patients with an early and late diagnosis regarding disease activity, function, metrology and radiological repercussion. This observational data suggests that early diagnosis may not confer a better prognosis. These results must be interpreted in the light of the cross-sectional, observational study design. It may be that early diagnosis is associated with more active disease, reducing the potential benefit of an early intervention rather than simply reflecting paucity of benefit of early treatment. On the other hand, many patients in this cohort were diagnosed as AS cases more than 10 years ago, when therapeutic strategies were clearly different from the ones that are available now.

In terms of therapeutic approach there are some interesting data for analysis. As usual a great proportion of patients are taking NSAIDs (79.1% daily or on demand). A lower but significant proportion of cases (17.6% in total and 12.5% in axial form) are taking corticosteroids. These results reinforce previous results from a preliminary study in Portuguese SPA [11] where corticosteroid prescription was considered higher than in other countries. Sulphasalazine is the most used DMARD (36.9%). TNF-α blockade (22%) is prescribed in a relatively small group of patients as in other studied populations [14, 21] and is used much less often than the estimated proportion of cases thought by expert opinion to warrant treatment with this specific therapy (30–49%) [22, 23]. The positive correlation between BASMI and anti-TNF-α therapy (p=0.024) may be an indirect evidence of the delay in starting biologic therapies in this cohort.

The mean BASDAI (4.2±2.3), BASFI (4.1±2.7) and BASMI (4.0±2.5) values were similar to values of other published cohorts [13, 21, 24].

Reference centile charts have been developed in British AS patients for BASDAI, BASFI and BASMI [8], but no published charts are available from any other population. Furthermore, there is little data about inter-ethnic differences in AS characteristics, and no reference centile charts have been published for mSASSS in any studied population. In constructing centile charts we tried different generalized linear models and selected the generalized linear model with Gaussian family distribution and identity link function (this is linear regression model) for the Bath AS indices and selected the generalized linear model with Gamma family distribution and log link function for mSASSS. In spite of using different statistical methodologies, the smaller sample size available to us and the differences in gender distribution between the two studies, our findings are very similar to those reported previously for the British population [8].

The charts do provide some descriptive information regarding disease activity, functional impairment, metrology and radiological impact in AS over time. These reference charts (Fig. 1), after proper validation, may be applied to compare the same population over time or

different populations. This visual representation may also improve patient understanding of the disease, which may improve patient compliance to treatment. Other potential benefits as their application on an individual basis would require the collection of longitudinal data and trials to confirm them. A potential weakness of the study is that the data is cross-sectional, and it is not yet known to what extent disease activity measures in individual patients track consistently relative to the overall patient cohort. Thus, longitudinal studies are required to determine if patients with high or low outcome measures at one point in time remain consistently high or low at other time points. The patient selection method is another potential limitation of the study, possibly affecting the extent to which the data reflects cases with AS overall in the general population, which may be milder than the clinical cohort studied here. However, as most AS patients in Portugal receive their care through outpatient clinics such as those studied here and in this study a high proportion of those clinics covering a range of demographic regions were included, we feel that this bias is likely to be minor.

Nonetheless, the analyses of BASDAI charts (Fig. 1) confirm the previous finding that AS remains active throughout the disease course [25]. Furthermore, as previously reported in English patients, women are more functionally impaired than men and have greater disease activity despite better metrology and, in our cohort, less radiological change. These results suggest once again differences between men and women in the AS phenotypic expression.

In conclusion, we have described the clinical profile of AS in Portugal, and simultaneously we have constructed the centile charts for BASDAI, BASFI, BASMI and mSASSS. A potential use of these charts is to show changes in the clinical profile of AS patients in Portugal over time due to either changes in treatment strategies or changes in the disease itself. An additional interest would be to facilitate comparisons between different populations.

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2.2. GENETIC DNA BASED STUDIES

2.2.1. State of the art in genetics of Ankylosing Spondylitis in 2007

Santos FP, Bastos E, Ligeiro D, Mourão AF, Chaves R, Trindade H, Guedes-Pinto H, Branco JC. **Genetic basis of ankylosing spondylitis**. *Acta Reumatol Port*. 2007 Jul-Sep; 32(3): 243-52.

ARTIGO DE REVISÃO

ESPONDILITE ANQUILOSANTE E SUA BASE GENÉTICA

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R. Chaves,** H. Trindade,*** H. Guedes-Pinto,** J. C. Branco*

Resumo

A Espondilite Anquilosante (EA) é uma doença reumática inflamatória frequente na qual o componente hereditário parece ser relevante. A importância dos factores genéticos radica em grande parte no complexo *major* de histocompatibilidade (MHC). A associação estreita com o grupo de alelos HLA-B*27 tem vindo a ser descrita, desde há cerca de 30 anos, de forma consistente em diferentes regiões do globo. Estudos recentes demonstraram, no entanto, que outros genes, do MHC e não pertencentes ao MHC, poderão também estar implicados, quer na susceptibilidade, quer nas manifestações fenotípicas da doença. Por outro lado, vários estudos têm sido realizados sobre as diferentes hipóteses explicativas da fisiopatologia da doença, o que tem contribuído para um melhor conhecimento das suas bases genéticas. Este artigo de revisão tenta sumariar o estado actual do conhecimento nesta área. Os novos dados encontrados poderão, num futuro próximo, contribuir para modificar a avaliação destes doentes e perspectivar novas abordagens terapêuticas.

Palavras-chave: Espondilite Anquilosante; Genética; Complexo *Major* de Histocompatibilidade (MHC); Susceptibilidade; Fenótipo

Abstract

Ankylosing spondylitis (AS) is a common rheumatic condition, highly heritable. Much of the genetic contribution to the disease lies in the major histocompatibility complex (MHC). The association with the allele group HLA-B*27 has been described worldwide for 30 years. On the other hand, genome wide scans have provided some interesting results showing that other MHC and non-MHC genes could be implicated either in disease susceptibility and phenotypic manifestations. Different hypothesis for disease pathophysiology have been investigated which contribute for a better understanding of the genetic basis of AS. This review aims to summarize the status of the knowledge in this exciting area. New data may, in a near future, change the screening of patients and generate new insights for the emergence of novel therapies.

Keywords: Ankylosing Spondylitis; Genetics; Major Histocompatibility Complex (MHC); Susceptibility; Phenotype

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Introdução

A Espondilite Anquilosante (EA) é uma doença que se inicia habitualmente na segunda ou terceira década de vida, caracterizada pela inflamação da coluna e das articulações sacro-ilíacas, com erosões e posterior anquilose. O envolvimento de articulações periféricas, ocorre em cerca de 40% dos casos. O processo inflamatório pode ainda ter uma expressão sistémica com envolvimento da *entesis*, uvea, aorta, pulmões e rins.[1] O início em idades jovens e o envolvimento sistémico são factores condicionantes de incapacidade, com importante repercussão em termos individuais e sociais.

A prevalência da EA nos caucasianos é de 0,1 a 0,9%, sendo a segunda artrite inflamatória mais frequente, após a artrite reumatóide.[2,3] Desconhece-se a exacta prevalência da doença em Portugal, embora uma publicação recente do «Observatório Nacional das Doenças Reumáticas» aponte para 0,6%, valor obtido a partir de um questionário em que foram avaliados 1.238 indivíduos.[4]

Os factores genéticos parecem exercer um papel essencial na doença, quer em termos de susceptibi-

lidade – a concordância em gémeos idênticos é superior a 90%,[5] quer em termos da sua actividade e da incapacidade funcional provocada – em que a repercussão é de 51% e 68%, respectivamente.[6] O Complexo *Major* de Histocompatibilidade (MHC) e o grupo alélico HLA-B*27 em particular, têm de forma continuada, vindo a ser referidos como conferindo uma forte susceptibilidade para a doença.[7-9] Estudos recentes de análise global do genoma e de *microarrays* confirmam a associação do MHC com a EA, mas apontam para a possibilidade de outros genes não pertencentes ao MHC conferirem susceptibilidade ou condicionarem a sua expressão fenotípica.[10-12] Não se pode ainda excluir a influência de outros factores entre os quais os ambientais.

Nesta revisão pretende-se descrever os principais genes, pertencentes ao MHC e não pertencentes ao MHC, que parecem estar potencialmente envolvidos na EA.

Os Genes do MHC

Todos os vertebrados possuem uma região no genoma designada Complexo *Major* de Histocompatibilidade (MHC). Em humanos o MHC está localizado no braço curto do cromossoma 6 (6p21.3) numa região muito densa em genes da resposta imunitária. Na figura 1, apresenta-se o mapa físico desta região, com os vários *loci* representados. Aqui encontram-se os genes que codificam para as proteínas dos antigénios de histocompatibilidade da classe I e II. Existe ainda uma região intermédia designada como MHC classe III, que contém genes com papel relevante no processo inflamatório. As moléculas da classe I e II apresentam regiões definidas por domínios polimórficos ($\alpha 1$, $\alpha 2$ e $\alpha 1$, $\beta 1$ respectivamente) e por domínios constantes ($\alpha 3$, e $\alpha 2$, $\beta 1$, respectivamente), como representado esquematicamente na figura 2. Uma perspectiva tridimensional das moléculas classe I está representada na figura 3. Estas moléculas têm um importante papel no processamento e apresentação de antigénios ao receptor das células T (TCR), permitindo a elaboração de respostas imunes antigénio específicas.

Cada classe do MHC é representada por mais de um *locus*, sendo designados, em *Homo sapiens*, de HLA (*Human Leucocyte Antigen*). Os principais *loci* da classe I são os HLA-A, -B e -C que são expressos na maioria das células nucleadas. Na classe II destacam-se os *loci* HLA-DR, -DQ e -DP (Figura 1) de expressão restrita a células B e apresentadoras «profissionais» de antigénios (células dendríticas de Langerhans).

HLA-B

De acordo com a informação de Janeiro de 2007, disponível na base de dados IMGT/HLA (www.ebi.ac.uk/imgt/hla), o *locus* HLA-B possui 851 alelos diferentes e entre eles destacam-se, pela sua correlação com a etiopatogenia da EA, os 43 subtipos pertencentes ao HLA-B*27. O alinhamento das

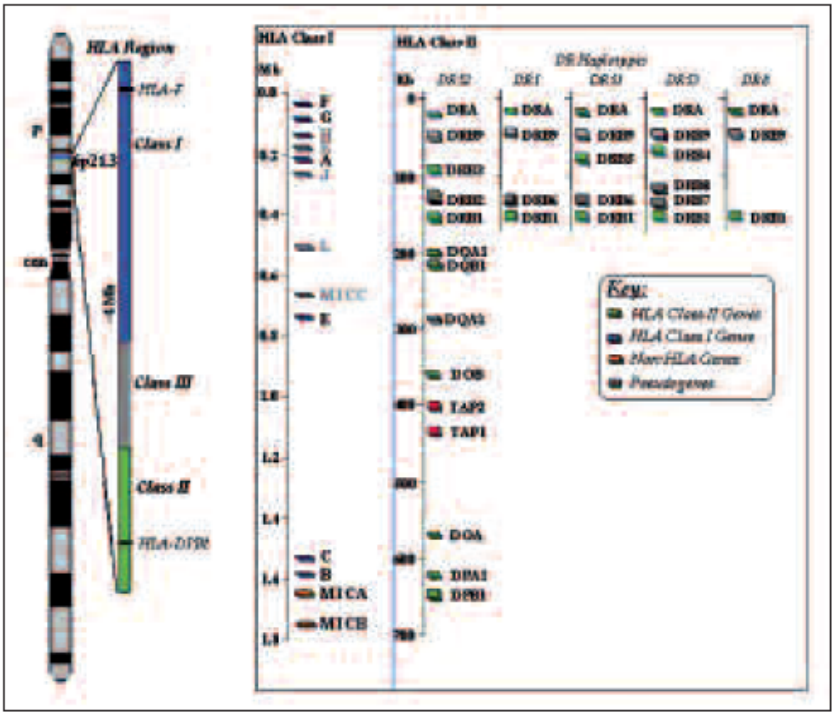

Figura 1. Representação das classes I, II e IIII do HLA, no cromossoma 6 (Adaptado de www.ncbi.nlm.nih.gov/projects/mhc).

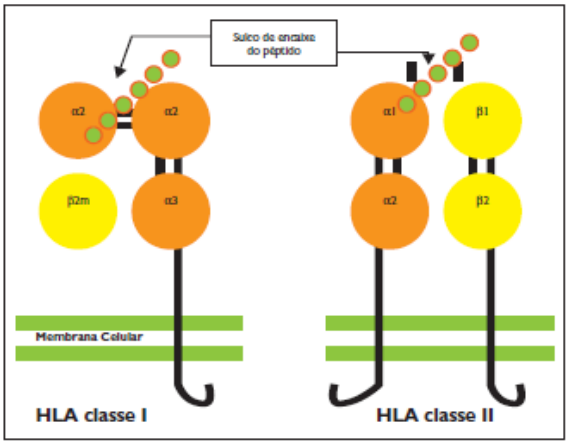

Figura 2. Representação esquemática das moléculas classe I e II do Complexo *Major* de Histocompatibilidade

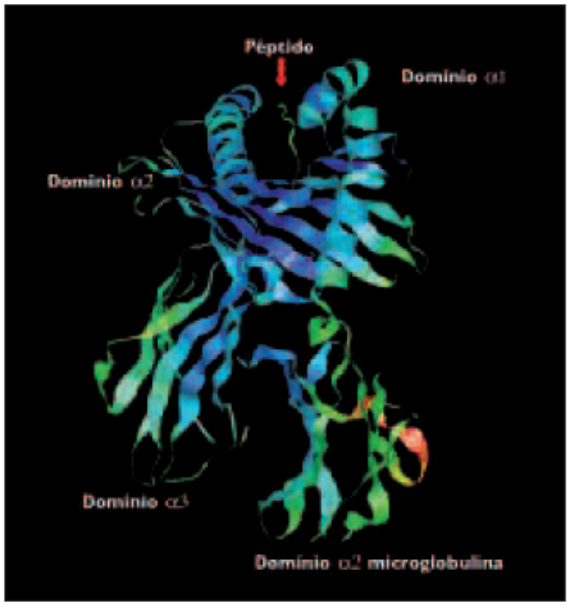

Figura 3. Estrutura de uma molécula de MHC de classe I ligada a um péptido. Imagem criada a partir do ficheiro 1HSA (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Domains) e recorrendo ao *software* RasMol (http://www.rasmol.org/).

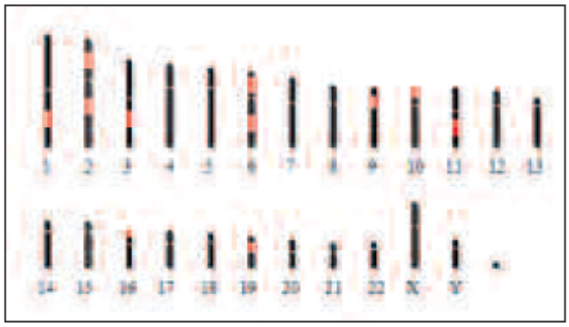

Figura 4. Representação esquemática das regiões cromossómicas identificadas como relevantes para a EA (adaptada de http://www.ncbi.nlm.nih.gov, com base nos artigos 10-12).

várias sequências permite identificar, em detalhe, as diferenças nos nucleótidos de todos os alelos.

Os diferentes subtipos do B27 têm uma distribuição populacional variável, encontrando-se o B*2705 na generalidade das populações. Os subtipos mais frequentes (HLA-B*2705, B*2702, B*2704, B*2707) conferem claramente susceptibilidade para a EA.[13] O B*2706, apenas identificado em algumas populações do Sudeste Asiático e o B*2709 descrito na população da Sardenha e em Italianos continentais, parecem pelo contrário, estar negativamente associados à EA.[14,15] A distribuição dos alelos B*2701, 10, 11, 12, 13 continua a ser desconhecida, não se sabendo se existe associação com a EA por serem raros.[16] Em Portugal, a avaliação da frequência das especificidades HLA (cf. Quadro I), numa população de doentes com EA (n=50) e numa população controlo de dadores voluntários de medula óssea (n= 174) revelou uma prevalência de B27 em 86% e 9,2% (p<0,0001), respectivamente. A distribuição das especificidades alélicas do HLA-B*27 parece porém ser similar nos dois grupos, sendo o alelo B*2705 o mais frequente. O facto de o alelo B*2707 ter sido detectado sómente na população livre de doença deverá ser comprovado na extensão do estudo (trabalho em decurso). Nos doentes portugueses com EA o B*27 é mais frequentemente veiculado pelos haplotipos A*2 B*27 Cw*2 DRB1*01 DQB1*05 (5.6%), A*24 B*27 Cw*2 DRB1*04 DQB1*03 (4.5%) e A*2 B*27 Cw*7 DRB1*13 DQB1*06 (4.5%). (dados não publicados).

O grupo HLA–B*27 parece ser, de facto, o mais relevante na susceptibilidade para a EA, mas muitas questões continuam por esclarecer. Tem sido apontado que mais de 95% dos indivíduos caucasianos com a doença, são HLA-B*27 positivos[17] com diferentes subtipos.[18,19] Curiosamente, na população geral, só 1-5% dos indivíduos HLA-B*27 positivos desenvolvem a doença e a sua presença, *per se*, não explica a recorrência em termos familiares.[20] O contributo do HLA-B*27 para a susceptibilidade parece oscilar apenas entre 20-50%.[5] Assim, a exacta função desta especificidade do HLA-B na fisiopatologia da entidade e o factor ou factores envolvidos na etiologia da EA, são aspectos que continuam a ser desconhecidos.

Quadro I. Distribuição da especificidade HLA-B*27 e alelos em doentes com EA e em controlos saudáveis portugueses

Frequência da especificidade HLA-B*27				
	EA N= 50 (%)	População Controlo N=174 (%)	p	OR (95% IC)
B*27+	43 (86)	16 (9,2)	p<0,0001	38,6 (16,7-89,3)

Distribuição de frequências dos alelos HLA B*27			
	EA N= 43 (%)	População Controlo N=16 (%)	p
B*2702	5 (11,6)	1 (6,3)	ns
B*2705	38 (88,4)	13 (81,3)	ns
B*2707	0 (0)	2 (12,5)	ns

A proteína expressa como HLA B27 apresenta propriedades estruturais semelhantes a outras moléculas da classe I, mas características únicas na ligação a péptidos. Comparativamente com outras moléculas da classe I, a proteína codificada pelo grupo de alelos B*27 é particularmente eficaz na apresentação de antigénios como foi demonstrada no *clearance* do vírus da Hepatite C[21] e na diminuição da progressão da infecção do vírus da Imunodeficiência Humana.[22] Esta função e os aspectos particulares que envolve a fase inicial da sua síntese, nomeadamente a tendência para o *misfolding*[23] e a formação de homodímeros,[24-26] são aspectos que poderão ter relevância na explicação dos mecanismos patogénicos em que se envolve. Estas e outras propriedades moleculares do B27 têm motivado a elaboração de diversas hipóteses explicativas do seu papel patogénico como a seguir se descrevem:

a) Péptido Artritogénico: A forte associação entre a EA e o grupo alélico HLA-B*27 suscitou a hipótese de que a proteína codificada pudesse, ela própria, ter um papel activo na fisiopatologia da doença. Este modelo é baseado na função habitual de apresentação de péptidos endógenos, pelas moléculas HLA da classe I, às células T.[13] A infecção por uma bactéria potencialmente patogénica levaria à ligação e apresentação de um «péptido artritogénico» pelo HLA B27. A proliferação de clones de células T $CD8^+$ motivariam a ocorrência de reacções cruzadas e o desencadear da doença.[13]

b) Mimetismo Molecular: Numa versão inicial apontava-se que anticorpos para antigénios estranhos ao *self* teriam uma reacção cruzada com a proteína HLA B27 o que promoveria o desencadear da doença.[27] Uma variante a esta formulação inicial é a teoria da homologia de péptidos em que um péptido artritogénico autólogo é produzido em níveis baixos. Um estímulo infeccioso poderia sensibilizar as células T que, por reacção cruzada com o péptido expresso em níveis baixos, desencadeariam uma resposta imune.[22,28]

c) Aumento do nível de expressão do HLA B27: Em ratos transgénicos para o HLA B27 e β2 microglobulina humana documentou-se que o grau de susceptibilidade para o desenvolvimento de espondiloartropatia se correlacionava com o nível de expressão do B*27, em termos de mRNA e de proteínas.[29] Em humanos a análise da distribuição dos diferentes alelos B*27 associados à doença revelou não existirem diferenças nas frequências destes entre doentes e a população saudável[30] embora a frequência de B*27 seja clararamente superior na população doente. Tal como nos modelos animais, foi descrita uma maior expressão do HLA B27 nos doentes com EA, comparativamente aos indivíduos saudáveis, pelo que se supôs que o nível de expressão do B27 à superfície das células pudesse ter um papel importante no processo imune.[31] Este aumento da expressão poderia determinar um aumento do *misfolding* de cadeias pesadas (da proteína expressa pelo HLA-B*27), uma sobrecarga no retículo endoplasmático, a activação do NF *k*B e assim a produção de citocinas pró-inflamatórias como o TNF-α e a IL-1.[32] Foi assim proposto que o aumento do nível de expressão do HLA B27 fosse considerado como um factor de susceptibilidade para a EA mas sem aparente influência nos índices de actividade ou gravidade da doença.[31]

d) Alteração do *Self*: O modelo de alteração do *self* prende-se com a alteração da cisteína (cys 67) da proteína B27. A formação de uma ponte bissulfito entre estes resíduos (cys 67) leva à formação de homodímeros de cadeias pesadas.[33] As condições que podem motivar a ocorrência destes homodímeros são o excesso de cadeias pesadas[34] ou a deficiência de β2 microglobulina, como acontece nos ratos transgénicos para o HLA B27 e para a β2 microglobulina humana.[35] Não se sabe como é que esta alteração poderá induzir a ocorrência de EA, sendo interessante avaliar a formação de homodí-

meros de cadeias pesadas nos diferentes subtipos de B27 (associados e não associados à doença).

e) Deposição de β2 microglobulina: A dissociação de B27 à superfície da membrana celular poderia motivar uma libertação crónica, em baixos níveis, de β2 microglobulina, que sendo captada pela sinovial, poderia conduzir a inflamação crónica.[36]

Outros genes do MHC

A combinação de determinados alelos da região do MHC numa frequência superior à que seria esperada, desequilíbrio de ligação, envolvendo os alelos HLA-B*27 e outros localizados nesta região, pode dificultar o estudo de genes de susceptibilidade a esta doença.[13]

O **HLA-B*60** foi indicado como aumentando em três vezes a probabilidade de desenvolvimento de EA nos indivíduos B27 positivos.[37] Hoje, pensa-se que este alelo ou outro em desiquilíbrio de ligação a ele associado, possa actuar de forma independente do B27 aumentando a susceptibilidade para EA.[38]

Os polimorfismos do promotor do gene do **TNF-α** (sobretudo o TNF -308 e o -238) têm vindo a mostrar resultados controversos. Em diversos estudos efectuados não se encontrou nenhuma associação entre os polimorfismos específicos do promotor do gene do TNF-α e a EA, mas em todos eles o número de doentes avaliados foi pequeno.[39-44] Em termos funcionais, foi descrito inicialmente uma significativa redução da frequência dos alelos -308 A e -238 A nos doentes B27 positivos, comparativamente à população controlo (indivíduos saudáveis, B27 positivos ou negativos).[42] Num estudo posterior, realizado em duas populações distintas (do sul da Alemanha e do Reino Unido), chegou-se a resultados diferentes. Na população alemã detectou-se uma redução significativa da frequência do alelo -308 A, mas não do alelo -238 A. Na população do Reino Unido não se verificou redução de nenhum. Conclui-se que estes polimorfismos não parecem exercer, por si só, um papel na susceptibilidade para a doença.[45] Um estudo realizado na população espanhola coloca no entanto a hipótese de que este polimorfismo possa influenciar a susceptibilidade para a doença nos indivíduos B27 negativos. O polimorfismo do TNF -238 A encontrava-se em 50% dos doentes B27 negativos, valor significativamente mais elevado que nos doentes B27 positivos e no grupo controlo B27 negativos.[46] Esta ideia foi posteriormente refutada por um estudo realizado na população holandesa no qual se mostrou que nem o polimorfismo -238 nem o -376 do TNF-α, se associavam à EA de forma independente do B27.[43] Na avaliação do possível contributo dos polimorfismos do gene do TNF-α para a susceptibilidade à EA, em função dos diferentes sub-tipos B27, conclui-se também não existirem diferenças entre doentes EA e indivíduos saudáveis B27 positivos. A maioria dos haplotipos HLA-B*27 estavam associados aos polimorfismos *wild type* do TNF-α (-238,2 e -308,2) e as suas frequências alélicas eram semelhantes na população de controlo (B27 negativa) e na população B27 positiva.[16] Finalmente a análise da influência dos polimorfismos do promotor do TNF-α nos níveis de transcrição,[47,48] conduziu a resultados muito contraditórios. Continua assim a ser difícil tirar conclusões àcerca da relevância deste gene na fisiopatologia da doença.

Algumas especificidades do **HLA classe II** têm também sido implicadas na susceptibilidade para a EA e eventualmente influenciar o fenótipo dos doentes. Os resultados obtidos revestem-se de alguma controvérsia. Uma significativa associação entre DRB1*01 e a EA e uma fraca, mas significativa, associação entre DRB1*08 (sobretudo nos indivíduos homozigóticos) e a EA, foi estabelecida independentemente do HLA-B*27.[7] O DRB1*01 parece ainda estar associado às formas esporádicas da doença, nos indivíduos HLA-B*27 negativos[49] o mesmo acontecendo para alguns polimorfismos do LMP2.[50] Em termos da sua influência ao nível fenotípico o polimorfismo LMP2 BB parece associar-se a maior probabilidade de ocorrência de uveíte anterior aguda;[51,52] os resultados são controversos em relação ao envolvimento articular periférico.[50,53] Se aparentemente não parecem existir diferenças nas frequências antigénicas dos doentes com envolvimento articular periférico ou axial, a maioria dos doentes com artrite periférica erosiva parecem ser DRB1*07 positivos. É assim possível que este alelo, ou outros estreitamente ligados, possa influenciar este tipo de envolvimento.[54] O HLA-DRB1*07 parece ainda associar-se a uma idade mais jovem para o início da doença,[7] sendo duvidoso se alguns polimorfismos do LMP2 exercerão também, neste aspecto, alguma influência.[55]

Genes Não – MHC Associados à EA

Estudos recentes, baseados na análise sistemática

do genoma humano, confirmaram a existência de *locus*, com possível relevância na susceptibilidade para a EA nas regiões cromossómicas 2p, 2q, 3p, 10q, 11p, 16q[10] ou 1p, 2q, 6p, 9q, 10q, 16q, 19q.[12] A região 16q tem-se evidenciado de forma universal nos vários estudos efectuados.[10,12,56] A figura 4 esquematiza as diversas regiões cromossómicas com relevância para a EA. Para além da susceptibilidade também as manifestações clínicas da EA poderão ter uma componente hereditária. Assim a actividade da doença foi associada a uma região no cromossoma 18p, a incapacidade funcional a uma região no cromossoma 2q, a idade para início da doença a uma região no cromossoma 11p[57] e a uveíte aguda anterior foi associada a uma região no cromossoma 9p[58] (cf. Quadro I). Estes dados são bons indicadores das regiões cromossómicas onde terá maior interesse a pesquisa de genes candidatos. Foram já identificados os genes MNDA (*myeloid nuclear differentiation antigen*- um modulador da transcrição e marcador de monócitos e macrófagos na inflamação crónica), o CXCR4/SDF-1 (*stromal-derived factor 1*- poderá constituir um potencial eixo pró-inflamatório na EA)[11] para além dos genes do complexo da IL-1,[59-62] e o do CYP2D6 (citocromo P450 debrisoquina 4-hidroxilase),[63,64] com associações confirmadas, e os do ANKH (*ANK, Mouse Homolog*),[65,66] MMP3 (*Matrix Metalloproteinase 3*),[67] TGF-β (*Transforming Growth Factor*),[68,69] TCR4 (*Toll-Like Receptor*),[12] com possível associação, à EA (cf. Quadro II).

Quadro II. Potenciais correlações existentes entre os diferentes segmentos cromossómicos e as manifestações fenotípicas da doença

Segmento Cromossómico	Fenótipo
cromossoma 18p	Actividade Doença
cromossoma 11p	Início em idade jovem
cromossoma 2q	Incapacidade Funcional
cromossoma 9p	Uveíte anterior aguda

Quadro III. Genes não MHC e EA

Consistentemente Associados	Inconsistentemente Associados
	IL-1R antagonista
CYP2D6	IL-6, IL-10
IL-1	TGF-β
	ANKH

O complexo do gene da **IL-1**, localizado no cromossoma 2p13, inclui os genes IL-1A e IL-1B (codificam as citocinas pró-inflamatórias IL-1α e IL-1β respectivamente) e IL-1RN (codifica uma molécula IL-1Ra com propriedades anti-inflamatórias que compete com as IL-1α e IL-1β) para além de mais seis genes. Assumem a seguinte ordenação do centrómero para o telómero, IL-1A, IL-1B, IL-1F7, IL-1F9, IL-1F6, IL-1F8, IL-1F75, IL-1F10, IL-1RN, ocupando uma região de ~360 Kb.[70] A importância das proteínas codificadas por estes genes na inflamação e na defesa do hospedeiro, fez com que diversos autores estudassem o seu papel na EA. Obtiveram-se resultados contraditórios de associações à EA para um número variável de sequências repetitivas dispostas em tandem localizadas no intrão 2[59,62,71] e para dois polimorfismos localizados no exão 6 do IL-1RN.[60] Um trabalho recente demonstrou que a IL-1B-511 e a IL-1F10-3 estão fortemente associadas com a EA, quer através de estudos de famílias quer de caso-controlo.[61]

Os polimorfismos do gene **MMP3**, cujo *locus* está localizado em 11q23, não parecem associar-se à EA, apesar deste gene se encontrar numa das regiões identificadas como relevante nos estudos de análise global do genoma e de se ter verificado que elevados níveis de expressão da MMP3 nas biópsias sinoviais se correlacionavam com maior actividade da doença.[67]

O gene codificante do **TGF-β** está localizado no locus 19q13.1. Esta citocina parece ter um papel crucial na neo-formação óssea pelo que o seu estudo detalhado, poderá abrir uma nova perspectiva terapêutica, no sentido de contribuir para evitar a anquilose. O polimorfismo TGF-β1 869 C foi associado a ossificação do ligamento longitudinal posterior na coluna cervical e com sindesmofitose num grupo de doentes Japoneses.[68,69] As variantes TGF-β1 713-8delC,[72] TGF-β1 869T[73] e TGF-β1 869 C[74] parecem associar-se a osteoporose, um problema relevante dos doentes com EA. No entanto, o estudo dos polimorfismos T869C e G915C não mostraram associar-se a maior susceptibilidade para a doença nem a início de sintomatologia em idade mais jovem.[75]

O gene **ANKH** está localizado no *locus* 5p14.1-p15.2. Recentemente, foi identificada uma proteína transmembranar, denominada ANK, que exporta pirofosfato inorgânico (ppi), um importante inibidor da calcificação, dos compartimentos

intracelular para o extracelular.[76] Uma mutação espontânea no *locus* da anquilose progressiva do rato (ank) origina nos homozigóticos uma forma progressiva de artrite generalizada acompanhada por deposição de mineral e formação aberrante de osso novo, resultando em anquilose e eventual destruição articular,[66] muito semelhante à EA do humano. Este gene foi implicado na condrocalcinose[77,78] (forma familiar autossómica dominante) (OMIM 118600) e na displasia craniometafisária[79,80] (forma autossómica dominante) (OMIM 123000). Várias variantes do ANKH foram associadas à susceptibilidade para EA,[76] contudo, os resultados necessitam de confirmação em amostras de maiores dimensões.[65] É possível que o ANKH não esteja apenas relacionado com a susceptibilidade mas também com a gravidade da anquilose óssea.

O gene **CYP2D6** está localizado no cromossoma 22q13.1. O polimorfismo CYP2D6*4 parece estar presente em, aproximadamente, 5 a 10% dos caucasianos, levando a um baixo metabolismo oxidativo dos fármacos (denominado «poor metabolizer», pm). Este fenótipo pode estar associado a pelo menos 15 variantes alélicas do CYP2D6, 75% das quais são CYP2D6*4 homozigóticos,[81] tendo sido associadas a diversas situações patológicas como a doença de Parkinson,[82] a neoplasias,[83] à esclerose sistémica[84] e ao lúpus.[85] Uma associação significativa foi observada entre homozigóticos e a EA. Pelo contrário, o risco de desenvolver a doença em indivíduos heterozigóticos não está aumentado.[63] O mecanismo de acção exacto não está claro, mas ilustra a importância que a incapacidade do metabolismo xenobiótico pode ter na fisiopatologia das doenças inflamatórias.

O gene **TLR4** está localizado no locus 9q32-q33. Alguns polimorfismos deste gene poderão estar associados à EA[12] assim como o **CD14-206T** (*cluster of differentiation 14*)[86-88] e uma mutação na posição 3020 do gene CARD15[89-92] a doenças intestinais inflamatórias. Estes dados colocam em evidência a função do sistema imune inato na patogenia desta doença. Esta evidência faz relançar a ideia de que os agentes infecciosos poderão, para além dos factores genéticos, exercer uma influência importante na patogenia da EA, à semelhança do que é conhecido para as artrites reactivas, embora nenhum agente tenha sido consistentemente associado à EA.[93] O CD14 e o TLR4 em conjunto com a proteína MD-2, fazem parte do complexo do receptor para os lipopolissacaridos (LPS- glocolipido específico das paredes celulares das bactérias gram negativas). A ligação de LPS ao receptor emite um sinal transmembranar que condiciona a activação do NF-*k*B e a consequente libertação de citoquinas pró-inflamatórias, com início do processo inflamatório.[94] Outros agonistas, bacterianos e do hospedeiro, poderão ser reconhecidos pelo TLR4, como a *C trachomatis* e a HSP60 (*heat shock protein*),[95] o que reforça a ideia de que a Resposta Imunitária Inata pode ter um papel crucial no desequilíbrio dos passos inicias da inflamação.

Conclusões

Os progressos na área da genómica humana, na análise integrada do genoma através de *microarrays* possibilitam hoje novas abordagens que muito poderão contribuir num futuro próximo para o esclarecimento do ou dos genes envolvidos na EA bem como do(s) seu(s) mecanismo(s) de acção.

Os diferentes genes poderão contribuir não só para a susceptibilidade como influenciar as manifestações e a gravidade da EA. A continuação da investigação nesta área abre perspectivas para que se possam vir a identificar os doentes com maior risco de progressão e incapacidade, bem como colocar em evidência novos alvos terapêuticos.

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2.2.2. Studies of MHC genes

2.2.2.1. HLA class I and II

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ABSTRACT

Introduction: HLA-B27 is known to be the major MHC gene, remarkably associated with Ankylosing Spondylitis (AS). Also, there is substantial evidence that other MHC genes, including HLA-B and non-HLA-B alleles, appear to be associated to AS. We aimed to investigate MHC class I and II alleles and extended HLA-B*27 positive MHC haplotype (HLA-A/B*27/C/DRB1/DQB1) associations with susceptibility to, and phenotypic features of AS in a case-control cohort.

Methods: Patients (n=369) with AS diagnosis, according to the modified New York criteria (1984), were recruited in several outpatient clinics. From this group, B27 genotype data were available in 355 subjects. A random group of HLA*B27 positive (n=188) patients and a random group of controls (n=189) were selected and typed for HLA class I and II by PCR-rSSOP. The extended HLA haplotypes were estimated by Expectation Maximization algorithm using Arlequin v3.11 software. Case-control comparisons were made by contingency table analysis for individual *loci* and estimated haplotypes. Associations between genetic and phenotypic characteristics were performed through linear and logistic binomial regressions using Stata10.1 software.

Results: In this cohort, 285:355 (80.3%) of AS patients were B27-positive. No association was found between other HLA *loci* polymorphisms and susceptibility to AS, but several associations were observed with the phenotypic features of the disease. DRB1*08 allele was identified as a risk factor for uveitis. In contrast DQB1*04 appears to provide protection in terms of disease activity, functional, metrological and radiological repercussion of AS. The haplotype

A*02/B*27/Cw*02/DRB1*01/DQB1*05 (p<0.0001; OR=39.06; CI 95% [2.34-651]) seems to be the only one conferring susceptibility to AS. Several haplotype associations were also found between the genetic and phenotypic features of the disease. However, the most robust association was seen with A*02/B*27/Cw*01/DRB1*08/DQB1*04, which seems to provide protection in terms of disease activity, functional and radiological repercussion.

Conclusion: In this AS population HLA-B*27 prevalence (80.3%) is low. An haplotype conferring susceptibility to the disease and another that plays a protective role in terms of disease activity and severity were described. The identification of genetic variants of target genes could be very useful to establish models with relevance in terms of susceptibility, prognosis and eventually therapeutic guidance.

Key Indexing Terms:

Ankylosing spondylitis

MHC

Alleles

Haplotypes

Susceptibility

Phenotypic features

INTRODUCTION

Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease that mainly affects the axial skeleton with a significant genetic component determining both susceptibility to, and severity of, the disease [1,2]. Since the 1970's, it has been known that HLA-B27, a major histocompatibility complex (MHC) gene variant, is strongly associated with the disease [3,4]. HLA-B27 is present all over the world, but with a very wide distribution among ethnic groups and geographic regions [5]. Approximately 8% of the population of Western Europe, and 10-16% of Scandinavian and Eastern Europeans carry the HLA-B27 allele [6]. In Europe there is a north-south gradient of HLA-B27 prevalence: in Chukchic Siberians 19-40%, in Ugro-Finnish 12-18%, in Southern Europeans 2-6% [6]. Although increasing the risk of AS by 50-100 times, only a small proportion of HLA-B27 carriers develop AS (1-5% in most series) [7]. Familial studies suggested that HLA-B27 contributes to approximately 20-50% of the global genetic risk for AS [2].

There is now substantial evidence that other MHC genes, including other HLA-B and non-HLA-B alleles, are associated with AS or influence its phenotypic characteristics, in several populations [2, 8]. Moreover, genes outside the MHC seem to be strongly implicated in the disease's etiology. The recent development of low cost, high accuracy, microarray-based SNP genotyping has led to a revolution in common and rare diseases genetics etiology. Since the Wellcome Trust Case-Control Consortium's (WTCCC) pioneering GWAS publications, several genes associated with AS have been identified. ERAP1, for example, was validated in a wide variety of ethnic groups, including

Portuguese, Chinese, Koreans and Hungarians [9-13]. Similarly IL23R has been replicated in Canadian and white European populations [9,14-18].

Studies of the MHC in AS are complicated by the strong association with HLA-B27, and the extensive linkage disequilibrium (LD) present across this region [19]. It is therefore difficult to determine whether a individual associations are driven by direct association, or LD [20]. Since HLA genes are often found together in populations in strongly preserved haplotypes (i.e., blocks of genes in linkage disequilibrium that are transmitted together), it can be difficult to dissect the genes responsible for such an association within the "predisposing" HLA haplotype. Nonetheless there is suggestive evidence from several studies that MHC genes and HLA alleles other than HLA-B27 are associated with AS, and may influence its clinical phenotype [20]. In this context an association between HLA-DRB1*01 and spondyloarthropathy has been reported among British and Mexican populations [21,22]. Also, an association between HLA-DRB1*15-B27 haplotype and ankylosing spondylitis has been described in Sardinia [20,23]. Another interesting association between HLA-DRB1*08 and juvenile ankylosing spondylitis has been identified in Norwegian and Mexican cases [24,25]. Such findings strongly suggest that both HLA-B27 and HLA-DRB1*08, either independently or as part of a haplotype, contribute to the genetic susceptibility of early onset disease [26]. Finally there is no consensus among an association between HLA-DRB1*08 and uveitis. An association has been found in a Japanese population [27] but negative findings have been reported in Norwegian and Mexican studies [24,25].

The aim of this study was to investigate the individual MHC class I/II alleles and extended MHC B27 positive haplotypes (HLA-A/B*27/Cw/DRB1/DQB1) distribution, and their association with susceptibility to and clinical expression of AS in a case-control cohort of Portuguese origin.

MATERIAL AND METHODS

Subjects: A total of 369 unrelated AS patients, according to the modified 1984 New York criteria, were recruited from several rheumatology outpatient hospital departments. Patients (n=188) were randomly selected from the group of individuals serologically defined as HLA-B27 positive, and were included in the study. HLA-B27 positive healthy controls (n=189) were randomly selected from the national bone marrow donor registry. All individuals included in the study were of Portuguese ancestry and came from mainland Portugal. This study was approved by the Ethics Committees of the involved centers, and written informed consent was obtained from the participants in this study.

Patients completed a questionnaire containing a self-assessment of clinical features, including the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and the Bath Ankylosing Spondylitis Functional Index (BASFI). Metrology was performed by one of the investigators (FPS), in order to obtain the Bath Ankylosing Spondylitis Metrology Index (BASMI). Age at disease onset was defined as the age at onset of clinical symptoms. Similarly, disease duration was defined as the period (years) after the onset of clinical symptoms. When available, cervical and lumbar X-rays were independently evaluated by two rheumatologists (FS, AFM), using the modified Stoke Ankylosing Spondylitis Spinal Score (mSASSS).

HLA typing: DNA extraction was performed from peripheral blood lymphocytes using standard techniques. All patients and controls were typed for HLA-A, -B, -

Cw, -DRB1 and -DQB1 by a bead array Polymerase Chain Reaction - Reverse Sequence Specific Oligonucleotide Probe (PCR-rSSOP) using a commercial kit manufactured by One Lambda (One Lambda, Canoga Park, Cal, 2009).

Statistical Analysis: Genotype, allele frequencies and extended MHC haplotypes (HLA-A/-B/-Cw/-DRB1/-DQB1) were calculated from direct counts. These frequencies were estimated through an iterative Expectation-Maximization (EM) algorithm using Arlequin v3.11 software. Case-control comparisons were made by contingency table analysis using Fisher's exact test with Bonferroni correction for *loci* and haplotypes. The odds ratio (OR) and its 95% confidence interval (95% CI) were calculated for each allele and haplotype in order to estimate the association's magnitude. Associations between genetic and phenotypic characteristics were performed by linear and logistic regressions, using Stata 10.1 software. Significance was set at $p < 0.05$.

RESULTS

A total of 369 unrelated patients were recruited. HLA-B27 data were available for 355 patients. In this cohort, 285:355 (80.3%) of the patients were HLA-B27 positive; 188 were randomly selected for further analysis. This group includes 118 (62.8%) men and 70 (37.2%) women (ratio 1.7:1) with a mean age of 45.1 (±13.5 SD) years (range 20-79 years) and mean disease duration of 11.8 (±10.5) years (range 0-46 years). Epidemiological data of these cases are summarized in Table 1.

Genetic Susceptibility for AS

HLA-A, -B and Cw Allelic analysis

Eighteen, 26 and 13, HLA -A, -B and -Cw alleles were identified. In general all the alleles were identified in both groups (AS and controls). In contrast, A*36, A*69, B*42, B*52 and B*78 alleles were only identified in controls. Also, the frequencies of HLA-A*31 and HLA-B*08 increased significantly in AS patients versus controls (P=0.004, OR=5.19, 1.49<CI<18.1 and P=0.003, OR=3.97, 1.47<CI<10.8, respectively). However, upon Bonferroni correction no statistically significant differences were found in the distribution of any allele. The HLA-A*02, -B*27 and -CW*02 alleles were the most prevalent in our population, with frequencies of 32.9% vs. 31.2%, 52.4% vs. 51.9%, 32.2% vs. 30.9% in AS patients and controls, respectively (data not shown).

HLA-DR and -DQ Allelic analysis

Thirteen HLA-DR and five HLA-DQ alleles were identified both in patients and controls. No significant differences were found between the two groups. Allelic frequencies for HLA-DRB1*04, HLA-DRB1*13 and HLA-DQ*03, the most prevalent in our population, in patients and healthy controls, were 17.6% vs 16.9%, 12.5% vs 17.2%, and 30.6% vs 31.8%, respectively (data not shown).

HLA-B27 Haplotype analyses

The most frequent B*27 positive haplotypes in AS affected cases were A*02/B*27/Cw*02/DRB1*01/DQB1*05 and A*02/B*27/Cw*01/DRB1*04/ DQB1*03 (both with the same frequency, 5.57%), and for controls A*02/B*27/Cw*02/DRB1*13/DQB1*06 (6.5%). Table 2 shows the relative frequencies of each significant B*27 carrying haplotype found in the Portuguese population. Several haplotypes showed a significant difference between AS patients and healthy controls. However, after Bonferroni correction only the A*02/B*27/Cw*02/ DRB1*01/DQB1*05 (p<0.0001, p_c<0.0004) haplotype remained significant. This haplotype was only identified in cases, consistent with a marked effect on disease-suscpeptibility.

Genetic correlations with AS Clinical features

As mentioned previously, associations between genetic and phenotypic characteristics were tested by linear and logistic regression methods. These analyses was corrected for gender, age, familiar history and disease duration. Several associations were identified. Table 3 illustrates the most robust associations for alleles and haplotypes (extensive data are available online). Considering individual markers, DRB1*08 allele was associated with increased

risk of uveitis, whereas in contrast, DQB1*04 seems to provide consistent protection in terms of disease activity (BASDAI), functional (BASFI), metrological (BASMI) and radiological severity (mSASSS). Extended haplotypes bearing those single HLA-alleles showed consistent associations with the single marker analysis. Association was seen with haplotype A*02/B*27/Cw*01/DRB1*08/DQB1*04, which seems to provide protection in terms of disease activity (BASDAI), functional (BASFI) and radiological repercussion (mSASSS).

DISCUSSION

Ankylosing spondylitis is a multigenic inflammatory disease strongly associated with HLA-B27. The distribution of this gene in Portugal ranges from 2.2% to 4.9%, depending on the geographic region [28], which is in agreement with the estimated prevalence for western European countries [6]. In our study, the prevalence of HLA-B27 in AS cases was 80.3%, similar to Greece (80.5%) [29], lower than Spain (94.3%) [30], but higher than in Turkish AS patients [31]. The objective of this study was to investigate other HLA AS predisposing genes and to identify the most probable B27 associated AS carrying HLA extended haplotypes.

Our study of HLA-B27 positive individuals, showed no significant statistically differences in the distribution of alleles among AS patients and healthy controls in the Portuguese population. In other words, no additional isolated HLA class I and II allele appears to increase susceptibility or provide protection against AS. This contrasts with some previously reported findings in AS, possibly related to different methodologies used (inclusion of patients with spondylarthritis rather than AS, B27 positive and negative cases and controls) and different ethnical backgrounds studied. In fact, HLA-B40, a putative secondary susceptibility allele to AS in Caucasoid populations [32], was not increased among the Portuguese AS patients similarly to the outcomes in the Basque population [33]. However, HLA-A9 (A*23 and A*24 allele groups) was significantly associated with AS in Basque but not in our population [33]. In addition, other B locus were identified as conferring susceptibility for SPA as HLA-B*15 and B*49 in Mexican populations [22] and HLA-B*14 in African populations (Togo and Cameroon)

[34, 35]. HLA-B*07 [36] and HLA-B*51 alleles have been shown to have a protective influence on the risk of developing AS in several Mediterranean populations [36-38].

Data regarding the effect of HLA-C locus in terms of susceptibility to AS are limited, however C*02 and C*06 were associated to AS in a Kuwait population study [39].

Finally, several class II associations with AS were found. HLA-DR*01 was described in association to AS independently of HLA-B*27 in British and Mexican populations [21, 22], HLA-DR*08 in British subjects [21]; a similar effect was described for HLA-DQ*02 in Spanish patients [40].

The density of genes and the complexity of the Linkage Disequilibrium (LD) in the MHC region, create challenges for the detection of AS-causative variants. Studying haplotypes may be a way to highlight these possible causative variants. That is the main reason to perform this extensive haplotypic analysis. In this study, the haplotype A*02/B*27/ Cw*02/DRB1*01/DQB1*05 was only observed in AS cases, conferring susceptibility to the disease.

To our knowledge this is the first study in AS in which HLA-A, -B, Cw, DR, -DQ haplotypes were studied simultaneously. This complicates the comparisons of results between studies. In Sardinia two different haplotypes were described as conferring susceptibility; A*02/B*2705/Cw*02/DR*02 [41], and A*02/B*27/Cw*02/DR*16 [20]. These are in accord with our studies with respect to HLA Class I associations, but differ at HLA Class II. Data regarding the involvement of HLA-DRB1 and DQB1 haplotypes in AS are limited.

Associations with B*27/DRB1*11/DQB1*03 in the Tunisian population [36] and B*27/DRB1*07 in the British population have been reported [42].
Considering HLA-B/-Cw, B*27/Cw*01 and B*27/Cw*02 have been described in association with AS in Caucasian populations [43-45]. Analysis in Mexican and Spanish patients including HLA-B27 subtype data analysis showed that B*2705/Cw*0102, B*2705/Cw*02022, and B*2702/Cw*02022 were associated with AS, and that B*2702/Cw02022 and B*2705/Cw02022 in Tunisians [45].
The HLA-B*27/Cw*02 association was also observed in the current study in the Portuguese population. Whilst there is some agreement between these studies, particularly at HLA-Class I loci, further research in adequate powered and homogeneous groups of patients using the same methodology will be required to achieve definitive conclusions.

Clearly, genetic factors play a crucial role in AS susceptibility. In addition to susceptibility, disease phenotype, as well as response to treatment may be related to genetic background. Recent studies have shown that clinical phenotypes of AS are probably related to genetic background [46]. Our study investigated the relationship between several MHC alleles and haplotypes and various clinical manifestations, including the age of symptom onset, family history, uveitis, BASDAI, BASFI, BASMI and mSASSS.
Many clinical features of AS are associated with the course of the disease and treatment. With this in mind, the analysis performed in our study tried to minimize this effect. Our results suggest that several alleles other than HLA-B27 may influence the clinical features of the disease. HLA-B*18 is associated with a younger age at onset, DQB1*02 with family history, and B*14, B*44, DRB1*08

with uveitis. In terms of activity and severity of the disease, HLA-DQB1*04 consistently seems to play a protective role, reflected in the lower values of BASDAI, BASFI, BASMI and mSASSS. Another interesting result is the risk conferred by B*14 and DRB1*08 for uveitis and radiological severity (higher values of mSASSS). With the exception of the association between HLA-DRB1*08 and uveitis, described previously by Brown et al. [34], none of these associations were previously reported in the literature. There is however support for these findings in the literature. HLA-DRB1 alleles were reported to influence the age of symptom onset in the Finnish population, with DRB1*08 being associated with younger and DRB1*03 with older ages of onset [26]. DRB1*08 was also observed in Mexican patients with juvenile onset disease [25]; HLA-DR*07 was described associated with younger age of onset [21,47]. Radiographic severity was related to the presence of HLA-B*4100, DRB1*0804, DQA1*0401, DQB1*0603 and DPB1*0202 [48].

There is a lack of information in the literature on the association between haplotypes and the clinical features of the disease. In our study, no haplotypes were associated with a family history or risk for uveitis. However, two haplotypes were significantly associated with younger age of onset (HLA-A*32/-B*27/-Cw*02/DRB1*15/-DQB1*02 and HLA-A*01/-B*27/-Cw*07/-DRB1*13/-DQB1*06), and two with later age of onset (HLA-A*26/-B*27/-Cw*02/-DRB1*11/-DQB1*03 and HLA-A*32/-B*27/-Cw*07/-DRB1*04/-DQB1*03). Moreover, the haplotype A*02/B*27/Cw*01/DRB1*08/ DQB1*04 seems to provide protection from the activity, functional severity, and radiological repercussion of the disease. Interestingly, it involves DQB1*04 allele, which as

an isolated allele confers "protection" for disease activity and severity, and also DRB1*08, which as isolated allele is related with an increased risk for uveitis and radiological severity. This observation may reinforce the possible gene-gene interaction phenomenon.

There were some limitations to this study. It only includes HLA-B27 positive patients and the size of the studied cohort is small. The statistical significance of the findings is not definitive, and would not survive strict Bonferroni correction for the number of markers and dependent variables studied. However this approach of statistical correction may be overly conservative for studies such as this, where numerous linked markers and traits are studied. Whilst this study does identify some potentially informative associations regarding AS-pathogenesis, in particular supporting the existence of non-HLA-B27 MHC associations with AS and its clinical manifestations, they require validation in further studies.

CONCLUSIONS

This study confirms previously known associations, and shows some new associations, between genetic factors and AS. An important issue identified in this study is that susceptibility and phenotypic aspects of the disease may be influenced by different genetic features. In this context A*02/B*27/ Cw*02/DRB1*01/DQB1*05 seems to confer susceptibility for AS and A*02/B*27/Cw*01/DRB1*08/ DQB1*04 seems to provide protection for the activity, functional severity, and radiological repercussion of the disease.

List of abbreviations

AS: Ankylosing Spondylitis

BASDAI: Bath Ankylosing Spondylitis Disease Activity Index

BASFI: Bath Ankylosing Spondylitis Functional Index

BASMI: Bath Ankylosing Spondylitis Metrology Index

EM: Expectation-Maximization

HLA: Human Leukocyte Antigen

LD: Linkage disequilibrium

MHC: Major histocompatibility complex

mSASSS: modified Stoke Ankylosing Spondylitis Spinal Score

OR: Odds ratio

PCR-rSSOP: Polymerase Chain Reaction - Reverse Sequence Specific Oligonucleotide Probe

Competing interests

The author(s) declare that they have no conflict of interest.

Authors' contributions

FMPS, HGP, JCB participated in the conception and design of the study.

FMPS, MM, AFM, CR, JC, HS, AB, FG, PP, FG, MC, ES, RAS contributing for primary data collection.

FMPS, MM, DL carried out the lab work, performed the data analysis and drafted the manuscript.

FMPS, JEF, HT, HGP, JCB revise critically the manuscript.

All authors read and approved the final manuscript.

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Tables

Table 1. Demographic and clinical characteristics of AS patients.

Legend: BASDAI - Bath Ankylosing Spondylitis Disease Activity Index; **BASFI -** Bath Ankylosing Spondylitis Functional Index; **BASMI -** Bath Ankylosing Spondylitis Metrology Index; **HAQAS** - Health Assessmment Questionnaire Ankylosing Spondylitis; **mSASSS** - modified Stoke Ankylosing Spondylitis Spinal Score.

Table 2. Distribution of haplotypes HLA-B27 to both groups in the study (AS and controls).

Legend: Rel freq - relative frequency; **OR** - odds ratio; **CI** - confidence interval.

Table 3. Association between alleles/haplotypes and clinical features (most interesting results).

Legend: Linear regression: coefficient (*p* value) for continuous variables; * Binomial Logistic regression: (*p* value) for categorical variable. **BASDAI** - Bath Ankylosing Spondylitis Disease Activity Index, **BASFI** - Bath Ankylosing Spondylitis Functional Index, **BASMI** - Bath Ankylosing Spondylitis Metrology Index, **mSASS** - modified Stoke Ankylosing Spondylitis Spinal Score.

Additional files

Supplementary Table 1

Supplementary Table 1

Title: HLA-A antigens in Portuguese AS patients and healthy controls

Supplementary Table 2

Title: HLA-B antigens in Portuguese AS patients and healthy controls

Supplementary Table 3

Title: HLA-Cw antigens in Portuguese AS patients and healthy controls

Supplementary Table 4

Title: HLA-DRB1 antigens in Portuguese AS patients and healthy controls

Supplementary Table 5

Title: HLA-DQB1 antigens in Portuguese AS patients and healthy controls

Supplementary Table 6

Title: Association between haplotypes and clinical features

Table 1: Demographic and clinical characteristics of AS patients

Clinical Features	Cases
Sex, M:F (%)	118:70 (62.8:37.2)
Age (Mean ± SD)	45.1 ± 13.5
Age of symptom onset (Mean ± SD)	25.8 ± 10.5
Age at diagnosis (Mean ± SD)	33.4 ± 12.0
Disease duration (Mean ± SD)	11.8 ± 10.5
Familiar history (n (%))	68 (37.4%)
BASDAI (Mean ± SD)	4.2 ± 2.3
BASFI (Mean ± SD)	4.1 ± 2.7
BASMI (Mean ± SD)	3.8 ± 2.5
HAQAS (Mean ± SD)	0.9 ± 0.6
mSASSS (Mean ± SD)	20.6 ± 23.7
VS (Mean ± SD)	20.5 ± 17.3
Uveitis (n (%))	65 (35.9%)

BASDAI - Bath Ankylosing Spondylitis Disease Activity Index; **BASFI** - Bath Ankylosing Spondylitis Functional Index; **BASMI** - Bath Ankylosing Spondylitis Metrology Index; **HAQAS** - Health Assessmment Questionnaire Ankylosing Spondylitis; **mSASSS** - modified Stoke Ankylosing Spondylitis Spinal Score.

Table 2: Distribution of haplotypes HLA-B27 to both groups in the study (AS and controls).

Haplotypes	Patients		Controls		Statistics		
A/B/Cw/DRB1/DQB1	n	Rel Freq (%)	n	Rel Freq (%)	*p value*	OR	[CI 95%]
02/27/01/04/03	18	5.57	14	4.33	0.476	1.307	0.64-2.67
02/27/02/13/06	15	4.64	21	6.50	0.393	0.706	0.36-1.39
24/27/02/04/03	14	4.33	12	3.72	0.695	1.180	0.54-2.59
02/27/02/01/05	18	5.57	0	0	**0.000**	39.06	2.34-651
02/27/01/07/02	6	1.86	0	0	**0.015**	13.28	0.75-237
31/27/02/07/02	6	1.86	0	0	**0.015**	13.28	0.75-237
02/27/01/08/04	5	1.55	0	0	**0.030**	11.21	0.62-204
02/27/02/07/02	0	0	10	3.10	**0.002**	0.047	0.00-0.80
02/27/01/01/05	0	0	10	3.10	**0.002**	0.047	0.00-0.80
68/27/02/04/03	0	0	6	1.86	**0.031**	0.076	0.00-1.36

Rel freq: relative frequency; OR: odds ratio; CI: confidence interval.

Table 3: Association between alleles/haplotypes and clinical features (most interesting results).

Alleles	Age of symptoms onset	UVEITIS*	BASDAI	BASFI	BASMI	mSASSS
A*24	-	-	-	-0.9 (0.035)	-0.3 (0.004)	-
A*66	-	-	4.0 (0.01)	-	-	-
B*14	-	(0.027)	-	-	-	1.0 (0.031)
B*18	-0.24 (0.006)	-	-	-	-	-
B*44	-	(0.02)	-	-	-	-
DRB1*08	-	(0.016)	-	-	-	0.8 (0.016)
DQB1*04	-	-	-2.2 (0.001)	-2.8 (p<0.000)	-0.4 (0.043)	-1.55 (0.003)
02/27/01/08/04	-	-	-2 (0.013)	-3 (0.010)	-	-1 (0.035)
32/27/02/15/02	-15 (0.019)	-	-	-	-	-
01/27/07/13/06	-23 (0.012)	-	-	-	-	-
26/27/02/11/03	17 (0.007)	-	-	-	-	-
32/27/07/04/03	24 (0.008)	-	-	-	-	-

Linear regression: coefficient (*p* value) for continuous variables; * Binomial Logistic regression: (*p* value) for categorical variable. BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, BASFI: Bath Ankylosing Spondylitis Functional Index, BASMI: Bath Ankylosing Spondylitis Metrology Index, mSASS: modified Stoke Ankylosing Spondylitis Spinal Score.

2.2.2.2. HLA class III

Sousa E, Caetano-Lopes J, Pinto P, Pimentel F, Teles J, Canhão H, Rodrigues A, Resende C, Mourão AF, Ribeiro C, Pinto TL, Rosa CM, da Silva JA, Branco J, Ventura F, Queiroz MV, Fonseca JE. **Ankylosing spondylitis susceptibility and severity--contribution of TNF gene promoter polymorphisms at positions -238 and -308.** *Ann N Y Acad Sci*. 2009 Sep; 1173: 581-8.

Ankylosing Spondylitis Susceptibility and Severity—Contribution of TNF Gene Promoter Polymorphisms at Positions –238 and –308

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Ankylosing spondylitis (AS) is a chronic inflammatory disease in which genetic factors play a central role. The efficacy of TNF blockers has reoriented research in this field in order to explain the influence of TNF in AS pathogenesis. The objective of this study was to access the influence of single nucleotide polymorphisms (SNPs) at positions –308 and –238 of the promoter region of TNF gene on AS susceptibility and prognosis. SNPs were determined by restriction fragment length polymorphisms in patients and controls. AS patients exhibited a decreased frequency of the A allele at position –238 (10%) when compared with controls (18%), suggesting that this could be a protective factor for disease susceptibility. In addition, the –308 GA/AA genotypes were associated with later disease onset in AS patients. These results suggest that TNF gene promoter polymorphisms at positions –238 and –308 could have a small influence on AS susceptibility and prognosis.

Key words: ankylosing spondylitis; TNF promoter polymorphisms; susceptibility; prognosis

Introduction

Ankylosing spondylitis (AS) is a chronic rheumatic disease in which genetic factors play a central role. Human leukocyte antigen (HLA)-B27 has been considered a major contributor for AS susceptibility and prognosis.

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However, genetic family studies in European descendent populations suggest that the contribution of HLA-B27 to the genetic risk for the disease may not be greater than 16%.[1] The genes of the major histocompatibility complex (MHC) as a whole are probably responsible for half of the disease susceptibility, suggesting that other genes inside MHC might also be relevant.[1]

Tumor necrosis factor (TNF) blockers have proven to be highly effective in improving AS manifestations, suggesting that TNF is at least as important to the inflammatory process of AS, as it is known to be in other rheumatic diseases.[2–4] This innovative concept has reoriented research aiming to elucidate AS disease pathogenesis and to identify potential markers of disease activity and prognosis. Several studies have identified increased TNF serum levels in AS patients, as comparing with controls, although this is not consensual.[5–8] In two of them a positive correlation was established between TNF serum levels and erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), and enthesitis.[6,7] Moreover, TNF mRNA has also been identified in sacroiliac joint of patients with AS.[9]

TNF production seems to be at least in part genetically determined and influenced by a wide range of factors, including transcription factors.[10] The TNF gene promoter is a highly polymorphic region, located in the short arm of the sixth chromosome, adjacent to the transcription start site. Single nucleotide polymorphisms (SNPs) are the most common mutations, and, although the majority is functionally inactive, some might influence the binding of transcriptional factors or modify the DNA spatial structure, thus regulating the gene transcription. For some of these SNPs, namely the ones at positions –238, –308, and –857, there is evidence that they are functionally active.[11,12] In addition to their possible role in TNF production, TNF gene promoter polymorphisms have been widely studied regarding their relevance to disease prognosis in various rheumatic conditions. In rheumatoid arthritis (RA), an association between the –238GG genotype, low functional status, and increased joint destruction has been depicted.[13,14] The –308GA/AA genotype was also found to be linked to an early disease onset, higher disease activity, and worse x-ray progression and functional class.[13,15] Interestingly, in juvenile idiopathic arthritis (JIA) this same genotype has been related to greater inflammatory activity demonstrated by an increased ESR and TNF serum levels, compared with controls.[16] Finally, in psoriatic arthritis (PsA), the –308GA/AA genotype was more frequent in patients with early disease onset and bone erosions.[17]

The fact that all these rheumatic diseases probably share similar inflammatory pathways, in which TNF seems to be a major determinant, coupled with their good response to TNF blocker, has made them recognized as TNF-dependent diseases. Therefore, it is possible that similar correlations between these polymorphisms, susceptibility, and severity might also be established in AS.

Material and Methods

Patients

Patients with the diagnosis of AS, according to the New York revised criteria,[18] followed in three Portuguese rheumatology centers (Hospital de Santa Maria, Hospital de Egas Moniz, and Hospital de São João) were randomly enrolled in this study, during a period of 2 years. Written informed consent was obtained from all patients. The study was approved by the ethics committees of the participating hospitals, and research was carried out in compliance with the Declaration of Helsinki. Patient data were collected using a detailed protocol that included information on age, gender, age of disease onset, disease duration, type of involvement, extra-articular symptoms (uveitis and enthesitis), axial mobility, TNF blocker treatment, ESR and CRP at the time of evaluation, and HLA-B27 typing. Disease activity, functional

capacity, and structural damage were determined and registered using the Portuguese versions of the BASDAI, the Bath Ankylosing Spondylitis Functional Index (BASFI), and the Bath Ankylosing Spondylitis Radiology Index (BASRI), respectively.[19–21] One hundred and seventeen healthy individuals were included as controls.

TNF Gene Promoter Polymorphisms

From each patient and control subject a blood sample was collected for determination of TNF gene promoter polymorphisms at positions –308 and –238.

Blood collected in EDTA-containing tubes was used for DNA extraction performed using the QIAmp DNA Blood Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations.

The TNF gene –308G/A and –238G/A polymorphisms were analyzed by restriction fragment length polymorphisms (RFLP). The forward primer contained one nucleotide mismatch, which allowed the use of the restriction enzymes *NcoI* and *RsaI*, respectively, for the detection of the –308G/A and –238G/A polymorphisms. PCR was performed in a 50-μL reaction mixture containing 100 ng of genomic DNA, 40 nM of each primer, 0.2 mM of each dNTP, 15 mM of $MgCl_2$, and 0.4 U of *Taq* DNA polymerase (ABgene, Epson, UK). The reaction mixture was incubated for 3 min at 95°C, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 60 s. Restriction with the enzymes (New England Biolabs, Hitchin, UK) was performed at 37°C, as described by the manufacturer.

Quantification of Serum TNF Protein

Serum samples were collected from patients and controls to determine TNF protein concentration by enzyme linked immunosorbent assay (ELISA).

TNF concentration was determined in serum samples, in duplicate, using the DuoSet ELISA kit (R&D Systems, Minneapolis, MN), as recommended by the manufacturer.

Statistical Analysis

Continuous variables were described as mean ± standard deviation. The Mann–Whitney *U* test was used to compare continuous variables, while Fisher's exact test and the chi-squared test were used to compare categorical variables.

As only one subject was found with the AA genotype in –238 position and two with AA at –308, this genotype was grouped with the GA genotype (GA/AA) for statistical analysis. Grouping genotypes did not change the conclusions. Conformance with Hardy–Weinberg equilibrium in the patients and control groups was performed using an exact test,[22] as implemented by Haploview software version 4.1.[23]

Statistical tests were considered significant when *P*-values were ≤ 0.05.

Results

One hundred forty-one Portuguese patients were evaluated: 68% males, mean age 44 ± 12 years, mean disease duration 20 ± 11 years, and mean age at disease onset 24 ± 9 years. Fifty-seven percent had axial, 4% peripheral, and 39% both axial and peripheral involvement. None of the patients had exclusive enthesopatic presentation, but 55% had concomitant enthesitis. In addition, 45% experienced at least one episode of uveitis. Regarding the genetic background, 90% of the patients were positive for HLA-B27, and a family history of spondyloarthritis was referred in 39% of the cases. Metrology measurements depicted a modified Schöber test of 2.8 ± 1.7 cm, a lateral spine flexion of 11.5 ± 6.6 cm, a chest expansion of 3.7 ± 1.6 cm, and an occiput-wall distance of 9.5 ± 7.8 cm. Cervical rotation was between 20° and 70° in 50% of the patients.

TABLE 1. Characterization of the Study Population

	Mean ±SD or % of patients
Age (years)	44 ± 12
Age at disease onset (years)	20 ± 11
Disease duration (years)	24 ± 9
Modified Schöber test (cm)	2.8 ± 1.7
Lateral spinal flexion (cm)	11.5 ± 6.6
Chest expansion (cm)	3.7 ± 1.6
Occiput-wall distance (cm)	9.5 ± 7.8
Cervical rotation >70°/20–70°/<20°	29%/50%/21%
Tender joints	1.3 ± 3.1
Swollen joints	0.6 ± 1.8
BASDAI	4.0 ± 2.2
BASFI	4.1 ± 2.5
BASRI	8.5 ± 3.8
ESR (mm/1st hour)	22 ± 18
CRP (mg/dL)	1.6 ± 2.7
TNF levels (pg/mL)	47 ± 55
TNF blockers	34%
Conventional disease modifying anti-rheumatic drugs	62%
Oral corticosteroids	31%
Non steroidal anti-inflammatory drugs	98%

SD = standard deviation; BASDAI = Bath Ankylosing Spondylitis Disease Activity Index; BASFI = Bath Ankylosing Spondylitis Functional Index; BASRI = Bath Ankylosing Spondylitis Radiology Index; ESR = erythrocyte sedimentation rate, CRP = C-reactive protein.

TNF blockers (infliximab 20% and etanercept 14% of patients); Conventional disease modifying anti-rheumatic drugs (sulfasalazine 93%, methotrexate 35%, azatioprine 2%, cyclosporin 2%, gold salts 2% of patients).

Thirty-four percent were undergoing treatment with TNF blockers, 20% were on infliximab, and 14% were taking etanercept (Table 1).

Regarding the TNF promoter polymorphisms at position –238, the A allele was detected in only 10% of the patients but in 18% of the controls ($P < 0.05$). The frequency of the alleles at position –308 was similar between patients and controls (Fig. 1). The distribution of genotypes in both positions, among HLA-B27-positive and -negative patients, was also not statistically significant.

In addition, the –238GA/AA genotype was associated with higher values of ESR (45.7 ± 28.8 mm/1sth) than was the –238GG genotype (20.2 ± 15.3 mm/1sthour) ($P < 0.05$). Also, patients with the –308GA/AA genotype were older at disease onset (28 ± 11 years) than those with the –308GG genotype (24 ± 9 years). TNF protein concentration was not detected in controls, while in patients, the mean TNF serum level was of 43 ± 50 pg/mL, and it did not differ between the genotypes. We did not find any other significant correlations between the genotypes at the –238 and –308 positions and clinical and laboratorial parameters or with TNF blocker treatment (Table 2).

Discussion

In this study we have found a decrease in the frequency of the A allele in AS patients comparing to controls, independent of HLAB27, suggesting a possible protective effect of the A allele at position –238. This result is supported by the findings of Höhler and colleagues, in a German population, in which there was a significant decrease of the A allele at position –238 in HLA-B27-positive AS patients relative to HLA-B27-positive and -negative controls.[24] Also, in Taiwanese patients, the frequency of the A allele was markedly diminished.[25] Other studies however did not confirm these results in South German, English, Mexican, Chinese, and Spanish populations.[26–29] In opposition, we did not detect any association between the polymorphisms at position –308 and AS in this group of Portuguese patients, which is concordant to what has been described in the Dutch, English, Spanish, and Mexican populations.[26,27,29–31] Some of these differences can be due to ethnic variations, but studies including a small number of patients are probably underpowered. Therefore, larger studies in different ethnic populations are required so that more reliable conclusions can be obtained. In addition, the systematic analysis of the human genome has recently highlighted the importance of several loci for AS susceptibility, which include the short arm of chromosome 6, where the TNF

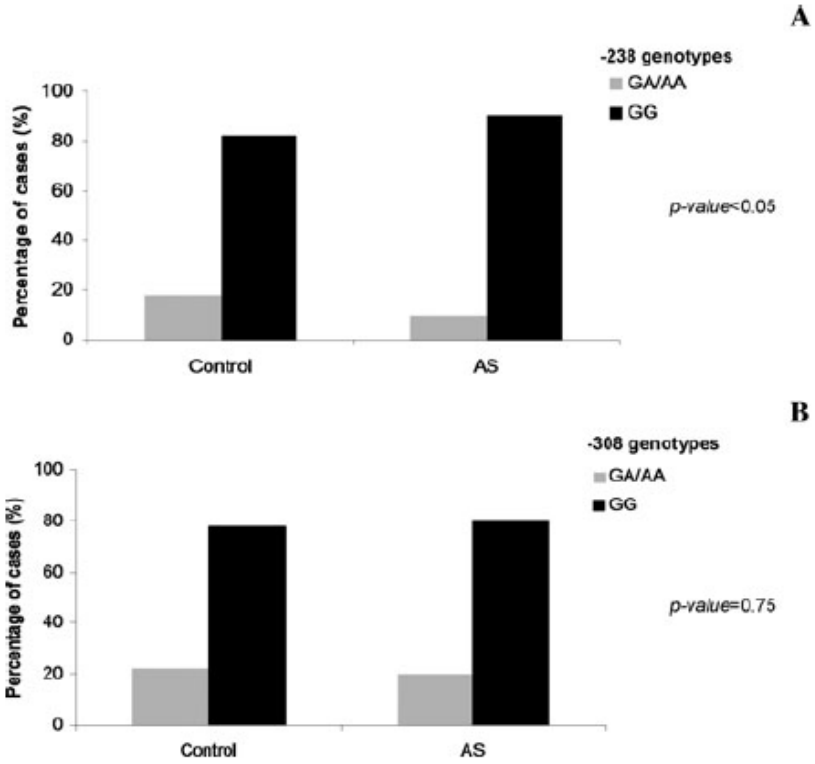

Figure 1. Distribution of –238 (**A**) and –308 (**B**) genotypes in healthy individuals and in ankylosing spondylitis patients.

TABLE 2. Distribution of Variables according to the Genotypes at Positions –238 and –308 (**P* < 0.05)

	–238 polymorphism		–308 polymorphism	
	GG	GA/AA	GG	GA/AA
Age (years)	44 ± 12	40 ± 13	43 ± 12	46 ± 12
Age of disease onset (years)	25 ± 10	20 ± 8	**24 ± 9***	**28 ± 11***
Disease duration (years)	20 ± 11	20 ± 13	20 ± 11	18 ± 12
Modified Schöber test (cm)	2.8 ± 1.7	2.9 ± 1.2	2.9 ± 1.7	2.7 ± 1.7
Lateral spine flexion (cm)	11.5 ± 6.6	11.4 ± 6.7	11 ± 6.7	13.4 ± 6.2
Chest expansion (cm)	3.6 ± 1.6	3.8 ± 2.0	3.6 ± 1.6	3.9 ± 1.6
Occiput-wall distance (cm)	9.7 ± 7.8	8.1 ± 7.9	9.8 ± 7.7	8.2 ± 7.7
Cervical rotation (<20°)	21%	17%	22%	20%
Tender joints	1.3 ± 3.2	0.9 ± 1.7	1.4 ± 3.2	1.0 ± 2.3
Swollen joints	0.6 ± 1.9	0.7 ± 1.4	0.7 ± 1.9	0.4 ± 1.1
Enthesitis	30%	25%	32%	22%
Uveitis	46%	36%	43%	54%
Anti-TNF treatment	35%	23%	35%	30%
BASDAI	4.1 ± 2.2	3.8 ± 2.6	4.0 ± 2.6	4.0 ± 2.3
BASFI	4.2 ± 2.4	3.7 ± 3.4	4.2 ± 2.4	3.9 ± 2.9
BASRI	8.5 ± 3.9	8.5 ± 3.3	8.8 ± 3.8	7.5 ± 3.9
ESR (mm/1st h)	**20.2 ± 15.3***	**45.7 ± 28.8***	23.2 ± 18.0	17.5 ± 16.0
CRP (mg/dL)	1.5 ± 2.6	2.3 ± 4.3	1.6 ± 2.8	1.8 ± 2.5
HLA-B27+	90%	83%	88%	96%
TNF levels (pg/mL)	46 ± 51	61 ± 103	42 ± 52	65 ± 63

gene and other HLA genes are located (1p, 2p, 2q, 3p, 6p, 9q, 10q, 11p, 16q, and 19q).[32,33] Studies like those from the TASC group (Australian, British, and North American investigators) might help to better explain the genetics of AS.[34]

The majority of the studies regarding TNF polymorphisms and AS have analyzed their possible contribution to susceptibility to the disease, while repercussions on disease prognosis have been less thoroughly investigated. Predicting the prognosis of AS patients has been difficult due to disease heterogeneity, its slow progression, and the absence of adequate outcome measures. Several factors are considered to be associated with a worse prognosis, including male gender, an early age at disease onset, peripheral arthritis particularly with hip involvement, the presence of extra-articular symptoms, and high BASDAI and BASFI scores, among others.[35] It is known that AS severity is largely genetically determined, and the outcome of a family member can be predicted by the disease pattern of previously affected members.[36] Therefore, considering the influence of some polymorphism of the TNF gene promoter on RA, PsA, and JIA severity,[13,16,17] it could be expected to have a similar effect in AS. In this group of Portuguese AS patients, the GA/AA genotype was associated with a later disease onset than the –308 GG genotype ($P < 0.05$). These data suggest that polymorphisms in this position could influence the disease course and, therefore, be a predictor of AS long-term prognosis. However, the –308 GA/AA genotype has been associated with higher TNF serum levels and higher disease activity in other inflammatory disorders. One possible explanation might be related to the observations made by Rudwaleit and colleagues suggesting that T cells in HLA-B27-positive AS patients and HLA-B27-positive healthy individuals have a decreased TNF production compared with HLA-B27-negative controls. According to this study, AS could be regarded has a low TNF/IFNγ disease and the decrease in TNF production as an additional risk factor for AS development. Furthermore, they also showed that –308GA/AA genotypes where associated with higher TNF production, which could be at least partially protective to AS.[8] This is in accordance with the decreased frequency of the A allele in AS patients identified in some studies.

The –238GA/AA genotype was associated with higher ESR values. This inflammatory parameter is a marker of disease activity in many other rheumatic diseases, but in AS it is reliable for only a subset of patients that have high ESR.[35] Therefore, the real impact of this observation for long-term outcome is not clear. Our observations suggest that the role of –238 and –308 TNF gene promoter polymorphism in AS susceptibility and prognosis is very small. However, this does not exclude that other SNPs in the same region, or haplotypes, might prove to influence AS course.

Conclusion

AS is a genetic-based disease, most certainly multigenetic, in which the risk for developing the disease is probably influenced by other factors besides the HLA-B27 gene. Results from this study suggest that TNF gene promoter polymorphisms at positions −238 and −308 could have a small influence on AS susceptibility and prognosis, although these data need further confirmation in a larger population. Finding genetic biomarkers of susceptibility and outcome in AS would be of help to clinicians in predicting which individuals need a more aggressive treatment to prevent disease progression.

Due to the length of this paper, we are unable to summarize all of the cutting-edge issues that surround this research. For this reason, we refer to the following recent literature on this subject.[34,37–39]

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Conflicts of Interest

The authors declare no conflicts of interest.

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2.2.3. Studies of non-MHC genes

2.2.3.1. *ERAP1* and *IL23R*

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Association of *IL23R* and *ERAP1* genes with ankylosing spondylitis in a Portuguese population

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Abstract

Objective

Association between ankylosing spondylitis (AS) and two genes, ERAP1 *and* IL23R, *has recently been reported in North American and British populations. The population attributable risk fraction for* ERAP1 *in this study was 25%, and for* IL23R, *9%. Confirmation of these findings to* ERAP1 *in other ethnic groups has not yet been demonstrated. We sought to test the association between single nucleotide polymorphisms (SNPs) in these genes and susceptibility to AS among a Portuguese population. We also investigated the role of these genes in clinical manifestations of AS, including age of symptom onset, the Bath Ankylosing Spondylitis Disease Activity, Metrology and Functional Indices, and the modified Stoke Ankylosing Spondylitis Spinal Score.*

Methods

The study was conducted on 358 AS cases and 280 ethnically matched Portuguese healthy controls. AS was defined according to the modified New York Criteria. Genotyping of IL23R *and* ERAP1 *allelic variants was carried out with TaqMan allelic discrimination assays. Association analysis was performed using the Cochrane-Armitage and linear regression tests of genotypes as implemented in PLINK for dichotomous and quantitative variables respectively. A meta-analysis for Portuguese and previously published Spanish* IL23R *data was performed using the StatsDirect® Statistical tools, by fixed and random effects models.*

Results

A total of 14 nsSNPs markers (8 for IL23R, *5 for* ERAP1, *1 for LN-PEP) were analysed. Three markers (2 for* IL23R *and 1 for* ERAP1*) showed significant single-locus disease associations, confirming that the association of these genes with AS in the Portuguese population. The strongest associated SNP in* IL23R *was rs1004819 (OR=1.4, p=0.0049), and in* ERAP1 *was rs30187 (OR=1.26, p=0.035). The population attributable risk fractions in the Portuguese population for these SNPs are 11% and 9.7% respectively. No association was seen with any SNP in LN-PEP, which flanks* ERAP1 *and was associated with AS in the British population. No association was seen with clinical manifestations of AS.*

Conclusions

These results show that IL23R *and* ERAP1 *genes are also associated with susceptibility to AS in the Portuguese population, and that they contribute a significant proportion of the population risk for this disease.*

Key words
Ankylosing spondylitis, *ERAP1*, *IL23R*.

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Conflict of interest: Dr M. Brown is currently applying for patents relating to the genes IL23R and ERAP1 in AS; the others authors declare no competing interests.

Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disorder with an estimated prevalence of 0.1–0.9% in Caucasian populations (1, 2). Although the contribution of the HLA-B27 allele to the overall genetic predisposition has been estimated at 20-30% and the contribution of all genes in the HLA region estimated at 40-50% (3), genes outside the major histocompatibility complex are strongly implicated in the aetiology of the disease. In particular, the genes *IL23R* and *ERAP1* have recently been demonstrated to be associated with AS in British and North American Caucasians (4). The population attributable risk fraction for *ERAP1* in this study was 25%, and for *IL23R*, 9% (4). Association of the *IL23R* findings has recently been confirmed in Canadian (5) and Spanish populations (6), but not yet been demonstrated for *ERAP1*. We sought to test the association between single nucleotide polymorphisms (SNPs) in these genes and susceptibility to AS in the Portuguese population. We also investigated the role of these genes in the pattern of AS clinical manifestations, including age of symptom onset and the Bath Ankylosing Spondylitis Disease Activity (BASDAI) (7), Functional (BASFI) (8) and Metrology (BASMI) (9) Indices, and the modified Stoke Ankylosing Spondylitis Spinal Score (10).

Methods

Subject

The study group comprised 360 unrelated AS patients and 285 ethnically matched healthy controls. Individuals included in the study were of Portuguese ancestry and came from mainland Portugal. All cases were diagnosed as having AS according to the modified New York Criteria (11). Cases were recruited from hospital outpatient departments; controls were healthy Portuguese bone marrow donors. This study was approved by the Ethics Board of the involved centres, and written informed consent was obtained from the individuals involved in this study.

Patients completed a questionnaire containing a self-assessment of clinical features, including the BASDAI and the BASFI. Metrology was performed by one of the investigators (FS), to obtain the BASMI. Age at disease onset was defined as the age at onset of clinical symptoms. Similarly, disease duration was defined as the period (years) after the onset of clinical symptoms.

Genotyping

Genomic DNA from cases and controls individuals was prepared from peripheral blood lymphocytes using standard techniques. Samples were genotyped for the 14 nsSNPs markers used in the Wellcome Trust Case-Control Consortium/Australo-Anglo-American Spondyloarthritis Consortium (WTCCC/TASC) study (4). Eight SNPs were typed in and around *IL23R* (rs1495965, rs10489629, rs11465804, rs10889677, rs1343151, rs1004819, rs11209026, rs11209032), 5 in *ERAP1* (rs2287987, rs30187, rs10050860, rs27044, rs17482078) and 1 in *LN-PEP* (rs2303138). Taqman® SNP genotyping assays assays (Applied Biosystems, Foster City, USA) were used for genotyping, which was performed according to the manufacturers protocols (see Table I).

Genotyping reactions were performed with an AB 7900HT, and the allele call by the analysis of allelic discrimination plots with AB SDS 2.3 software. Replicate genotype known and negative control samples were typed in each 96 well plate.

Statistical analysis

SNP genotype data was assessed for missingness (overall, and differences , cases and controls assessed by χ^2 test) and for Hardy-Weinberg equilibrium in controls. Individuals with >10% missingness were excluded (n=5 controls, 2 cases).

Association analysis was performed using the Cochrane-Armitage test as implemented in PLINK (12). Association between SNPs and the quantitative variables age of symptom onset, BASDAI, BASFI, BASMI and mSASSS were tested by linear regression assuming an additive model using PLINK, taking into account gender and disease duration as covariates.

Imputation analyses were carried out using Markov Chain Haplotyping software

Table I. *IL23R*, *ERAP1* and *LN-PEP* genetic variants analysed in AS patients and controls. Positions are given as per dbSNP build 129.

Genes	Chromosome	Position (bp)*	Gene location	Taqman ASSAY ID	NCBI SNP Reference
IL23R	1	67442801	Intron	C_1272321_10	rs1004819
	1	67460937	Intron	C_30279129_20	rs10489629
	1	67475114	Intron	C_31222838_10	rs11465804
	1	67491717	Intron	C_8367043_10	rs1343151
	1	67497708	Exon	C_11283764_10	rs10889677
	1	67478546	Exon	C_1272298_10	rs11209026
	1	67526096	Intergenic	C_8361864_10	rs1495965
	1	67512680	Intergenic	C_2720238_10	rs11209032
ERAP1	5	96150086	Intergenic	C_3056885_10	rs30187
	5	96147966	Intergenic	C_3056876_10	rs10050860
	5	96155291	Intergenic	C_3056893_10	rs2287987
	5	96144608	Intergenic	C_3056870_10	rs27044
	5	96144622	Intergenic	C_3056871_10	rs17482078
LN-PEP	5	96376466	Missense Mutation	C_25649482_10	rs2303138

Table II. Characteristics of the Portuguese AS cases and controls*.

	AS patients (n= 358)	Controls (n=280)
N° (%) males/ N° (%) females	226 (63%) / 132 (37%)	127 (44.6%)/158 (55.4%)
Age, years	45.4 ± 13.3	35.9 ± 11.1
Disease duration, years	19.1 ± 12.6	
BASDAI	4.2 ± 2.3	
BASFI	4.1 ± 2.7	
BASMI	4.0 ± 2.5	
mSASSS	20.9 ± 22.9	

*Except where indicated otherwise, values are the mean ± SD (standard deviation). AS- Ankylosing spondylitis, BASDAI- Bath AS Disease activity Index; BASFI- Bath AS Functional Index; BASMI- Bath AS Metrological Index.

Table III. Frequency of *ERAP1* and *LNPEP* minor allele frequencies in the Portuguese AS cohort.

Gene	SNP	Minor Allele	Portuguese Cohort			
			Case MAF	Control MAF	OR	*p*-value
ERAP1	rs27044	G	0.37	0.32	1.26 (1.10-1.60)	0.044
ERAP1	rs17482078	T	0.16	0.19	0.78 (0.58-1.05)	0.096
ERAP1	rs10050860	T	0.16	0.20	0.76 (0.57-1.01)	0.057
ERAP1	rs30187	T	0.47	0.41	1.26 (1.01-1.57)	0.035
ERAP1	rs2287987	C	0.16	0.20	0.77 (0.58-1.03)	0.074
LNPEP	rs2303138	A	0.08	0.07	1.23 (0.80-1.88)	0.33

(MaCH; http://www.sph.umich.edu/csg/abecasis/MACH/) using phased data from CEU individuals from release 22 of the HapMap project as the reference set of haplotypes. We only analyzed SNPs that were either genotyped or could be imputed with relatively high confidence (R^2>=0.3).Association analysis of imputed SNPs was performed assuming an underlying additive model using the software package MACH2ASSOC (Li, Willer, Ding, Scheet and Abecasis, unpublished data) which accounts for uncertainty in prediction of the imputed data by weighting genotypes by their posterior probabilities.

A meta-analysis study was performed using StatsDirect® software (13), specifically to test the association of *IL23R* and in the Iberian population, combining the Portuguese data presented here, and previously published Spanish data (6). Both fixed and random effects analysis was performed; non-combinability of studies was assessed using the Cochrane Q statistic, and the extent of heterogeneity between studies assessed using the I^2 statistic. Power calculations were performed using the Genetic Power Calculator (14).

Results

The Portuguese AS cohort population (Table II) included 228 (63.5%) men and 132 (36.5%) women with a mean age of 45.4 (±13.2 SD) years (range 20-79 years) and a mean disease duration of 19.1 (±12.6) years (range 0-60 years), of whom 82% were HLA-B27 positive. Epidemiological data of the cases and controls are summarized in Table II.

All the studied genetic markers were in Hardy-Weinberg equilibrium in the controls group, had missingess rates <10%, and none had differential missingness in cases and controls ($p<10^{-2}$). The minor allele frequencies (MAF) of the 14 SNPs are presented in Tables III and IV.

Single-marker association tests revealed significant AS associations for the *ERAP1* SNP rs30187 (odds ratio (OR) 1.26, p=0.035) and rs27044 (OR 1.26, p=0.044) (Table III). Unlike in the previous British and North American studies, no association was seen with the SNP rs2303138, which lies within *LN-PEP*, which flanks *ERAP1*. Of the 119 SNPs imputed in and around *ERAP1*, 32 demonstrated nominal association with AS (p<0.05), with the strongest association being with rs41135 (p=0.014) (Fig. 1).

Two SNPs in and around *IL23R* demonstrated significant association (rs1004819 OR=1.44, $p=4.9\text{x}10^{-3}$; rs10889677 OR=1.41, $p=5.8\text{x}10^{-3}$) (Table IV). The strongest associated SNP reported in both Crohn's disease (15) and psoriasis (16) (Arg381Gln; rs11209026) did not show any protective effect in our population. No imputed SNP was more strongly associated with AS than these two genotyped SNPs, but many SNPs in a block extending from rs10889667 as far as rs11465817 (67493685 bp from the p-telomere) were associated with AS with p<0.01 (Fig. 2). Of the 49 imputed SNPs in and around *IL23R*, 23 demonstrated nominal association with AS (p<0.05).

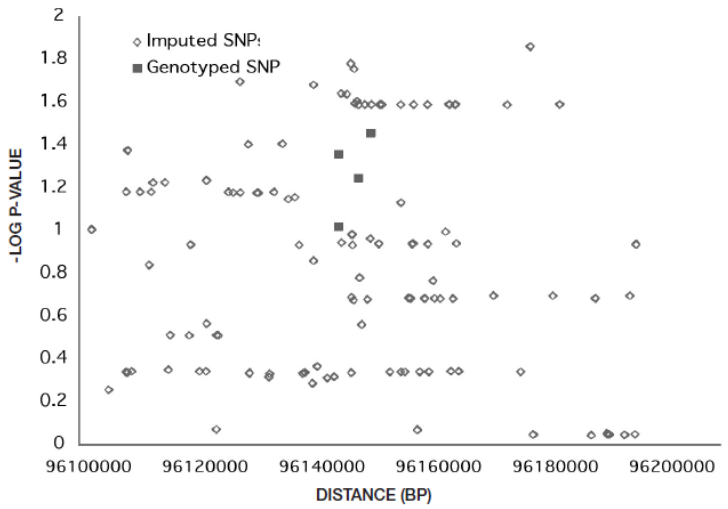

Fig. 1. Association results for imputed and directly genotyped SNPs in *ERAP1*. Distances are in base pairs (bp) from the p-telomere. Association significance is reported as -$\log_{10}$(*p*-values).

The population attributable risk fraction in the Portuguese population for rs1004819 is 11%, and for rs30187 is 9.7%.

The meta-analysis performed between Portuguese and Spanish populations (Table V) revealed significant AS associations, through the fixed effects, for SNPs rs1004819, rs11209026, and rs1343151. However, for markers rs11209026, rs1343151 and rs210889617 in particular, there were significant differences in the findings in the Spanish and Portuguese populations, reflected by significant Cochrane Q statistics (rs11209026, p=0.017; rs1343151, p=0.023; rs10889617, p=0.0005), suggesting that a random effects model should be applied. Considering the random effects models, the associations with AS remain significant for rs1004819.

No association was observed between *IL23R* or *ERAP1* variants and age of symptom onset, BASDAI, BASFI, BASMI or mSASSS (data not shown). The study had 80% power to detect associations with these quantitative variables at a significance level of α=0.05 for SNPs contributing >4% of the trait variance, assuming linkage disequilibrium between the marker and disease-associated variant of D'>0.8 and that the marker and disease-associated allele frequencies are equal. Considering the case-control analysis of disease susceptibility, assuming a population prevalence of disease of 0.4%, minor allele frequencies of 0.1–0.5, and D'>0.8, the study had 80% power to detect an additive association with heterozygote odds ratio of 1.6-1.8.

Discussion

Many candidate genes outside the MHC have been evaluated in different studies regarding AS susceptibility and/or phenotype associations. Recently, association has been demonstrated and confirmed with SNPs in and around the genes *ERAP1* and *IL23R* in British and North American populations (4).

The *IL23R* association with AS was also recently replicated in the Canadian (5) and Spanish (6) populations. The present study has replicated this association in the Portuguese population. The peak association in our cohort is seen with rs1004819. This is different from the UK, United States, Canada and Spanish data sets, where the peak association was observed for different SNPs, although the minor allele frequencies (MAF) that we have observed for SNPs in Portuguese were similar to those reported in other British and North Americans. Furthermore, the association observed in the Portuguese population had a similar magnitude of effect to the one described in those other populations, as can be appreciated by the attributable risk for rs1004819, which is very similar to the one reported for the most strongly associated SNP (rs11209032) in the British/North American populations (4). Interestingly, no association was established with rs11209032 in Portuguese or Spanish populations, which was strongly associated with AS in other Caucasian populations (4). Consistent with the Alberta

Table IV. Frequency of *IL-23R* minor allele frequencies in the Portuguese and Spanish cohorts.

Gene	SNP	Minor Allele	Portuguese Cohort				Spanish Cohort			
			Case MAF	Control MAF	OR	*p*-value	Case MAF	Control MAF	OR	*p*-value
IL23R	rs1004819	A	0.35	0.27	1.44 (1.13-1.84)	0.0049	0.32	0.29	1.21 (0.99-1.33)	0.076
IL23R	rs7517847	C	-	-	-		0.38	0.40	0.93 (0.76-1.13)	0.45
IL23R	rs10489629	C	0.43	0.45	0.93 (0.74-1.16)	0.51	0.42	0.46	0.92 (0.71-1.17)	0.50
IL23R	rs11465804	G	0.06	0.05	1.11 (0.68-1.78)	0.68	-	-	-	-
IL23R	rs11209026	A	0.04	0.04	0.98 (0,57-1.7)	0.95	0.03	0.07	0.46 (0.28-0.75)	**$1x10^{-3}$**
IL23R	rs1343151	A	0.33	0.34	0.93 (0.74-1.18)	0.56	0.29	0.38	0.68 (0.55-0.83)	**$2x10^{-4}$**
IL23R	rs10889677	A	0.28	0.36	1.41 (1.11-1.79)	0.0058	0.31	0.35	0.81 (0.65-0.99)	**$3.9x10^{-2}$**
IL23R	rs11209032	A	0.35	0.32	1.15 (0.91-1.45)	0.26	0.32	0.31	0.95 (0.77-1.16)	0.62
IL23R	rs1495965	C	0.45	0.45	0.98 (0.78-1.22)	0.84	0.44	0.43	0.96 (0.79-1.16)	0.69

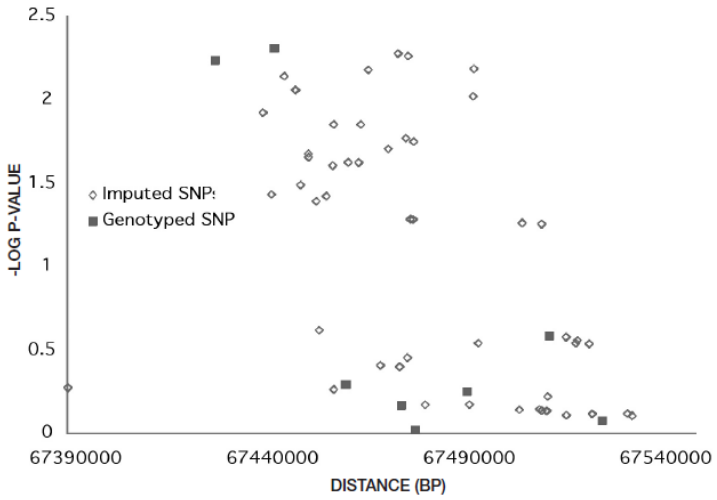

Fig. 2. Association results for imputed and directly genotyped SNPs in *IL23R*. Distances are in base pairs (bp) from the p-telomere. Association significance is reported as $-\log_{10}(p\text{-values})$.

Table V. Meta-analysis findings of combined Portuguese and Spanish studies of *IL23R*.

			Fixed effects		Random effects	
	Cochran Q *p*-value	I^2	OR	*p*-value	OR	*p*-value
rs1004819	0.28	0.16	1.3 (1.11-1.52)	1.2×10^{-3}	1.3 (1.10-1.55)	2.7×10^{-3}
rs10489629	0.61	0	0.89 (0.76-1.03)	0.12	0.89 (0.77-1.03)	0.11
rs11209026	0.017	0.83	0.59 (0.41-0.85)	3.1×10^{-3}	0.62 (0.26-1.5)	0.29
rs1343151	0.023	0.81	0.76 (0.65-0.89)	4×10^{-4}	0.77 (0.54-1.1)	0.16
rs10889677	0.0005	0.92	1.02 (0.87-1.19)	0.81	1.06 (0.62-1.83)	0.83
rs11209032	0.59	0	1.09 (0.94-1.27)	0.27	1.09(0.94-1.27)	0.27
rs1495965	0.68	0	1.01(0.87-1.17)	0.90	1.01 (0.88-1.17)	0.87

Canadian population, but in contrast to the British, United States, Spanish and Canadian (Toronto and Newfoundland) populations, the present study did not demonstrate any protective effect against AS for the Arg381Gln SNP (rs11209026) in the *IL23R* gene. This apparent lack of concordance may be due to the different ethnical backgrounds studied, or represent a type II error, given the modest power of the current study to detect association with this rare, protective, SNP. Given that significant association was seen with other SNPs in the Portuguese population, this suggests that rs11209026 and rs11209032 may not be the principal associated variants responsible for the *IL23R* association with AS, at least in the Iberian population. The imputed SNP data demonstrates that a broad region of *IL23R* is associated with AS in Portuguese. Association peaked in the 66kb region from rs10889667 to rs11465817, with a rapid reduction in strength of association outside of that region, suggesting that the primary associated variant(s) lies in that interval. Differences are apparent between our Portuguese data, and the previously reported Spanish data, as reflected by the significant Cochran's Q statistic in the meta-analysis of *IL23R* SNPs. Whilst statistically significant, with one exception (rs10889617), the magnitude of the heterogeneity is small, reflected by the low values of the I^2 statistic. Therefore it is not entirely clear as to whether a fixed- or random- effects model should be applied, with the latter being more conservative and less powerful. Considering the random effects, just 2 SNPs revealed association, with peak association for rs1004819 (Table IV and Fig. 1). The findings in the Portuguese population for rs10889677 were very similar to those observed in both the discovery and replication set of the study by the WTCCC/TASC in the British and North Americans (4), and in marked contrast to those reported in the Spanish population (6). We observed a MAF in cases and controls of 34% and 28% for this SNP respectively, compared with 31% and 35% in the Spanish population. In the WTCCC/TASC study, the MAF for this SNP in cases and controls respectively were 36% and 31% in the discovery cohort, and 37% and 29% in the control cohort. Association was seen between rs10889677 and AS in the Spanish study (OR=0.81 with minor allele 'A', P=0.039), but curiously it was in the opposite direction to the two cohorts reported by the WTCCC/TASC study, and our own Portuguese study.

Numerous studies have demonstrated association of *IL23R* SNPs with susceptibility to Crohn's disease (CD) (17), as well as to psoriasis (18) and psoriatic arthritis (19-21). *IL23R* therefore seems to be a common susceptibility factor for the major seronegative diseases, at least partially explaining their co-occurrence. In contrast, *ERAP1* is not associated with inflammatory bowel disease, and it is unknown whether it is associated with psoriasis or psoriatic arthritis. Whether *IL23R* or *ERAP1* polymorphisms influence clinical manifestations of disease such as age of symptoms onset, disease activity or severity is unknown. In the current study no association was observed with these traits, but the study power was only adequate for large genetic effect sizes (>4% of the trait variance).

This study confirms the association of *ERAP1* variants with AS, with a similar magnitude of effect to that seen for *IL23R*, as assessed by the population attributable risk fraction. We are not aware of other papers that have replicated this finding in populations other than British or North Americans. The strongest associated in *ERAP1* was rs27044 (OR=1.29, 95% CI=1.02-1.63; *p*=0.032). In contrast with *IL23R*, the association of *ERAP1* seems to be confined to AS. No association was observed between *ERAP1* SNPs and either Crohn's disease or ulcerative colitis in

the WTCCC/TASC study (4). Whether *ERAP1* SNPs are associated with psoriasis or psoriatic arthritis is unknown. No association was seen with the marker rs2303138 lying in *LNPEP*, providing further support to the hypothesis that at least a component of the association observed between this SNP and AS previously reported in British Caucasians is due to linkage disequilibrium with *ERAP1* polymorphisms.

The primary associated variant(s) in *ERAP1* remain uncertain. In this study, nominal association was seen between the SNPs rs26509 (96108436 from the p-telomere) and rs190298 (96191986 from the p-telomere), an interval of 84 kb. Very broad association was seen in the WTCCC/TASC study as well. Further fine-mapping and resequencing studies will be required to narrow the associated region and identify the key associated variant(s) to inform more targeted functional analysis of the mechanisms of involvement of this gene and AS.

Conclusions

These results show that *IL23R* and *ERAP1* genes are also associated with susceptibility to AS in the Portuguese population, and that they contribute a significant proportion of the population risk for this disease.

Authors' contributions

FPS, DL, HT, HGP, MAB and JCB participated in the design of the study. Experiments were performed by FPS, DL and MM. Statistical analysis was carried out by FPS and MB. FPS, AFM, ES, PP, AR, MS, AB, FG and MC contributed by providing human samples. Analysis of data was carried out by FPS, DL, MM and MB. Intellectual contributions to the manuscript were provided by FPS, JEF, HGP, MAB and JCB. All authors read and approved the final manuscript.

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2.2.3.2. *ANKH*

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ANKH and Susceptibility to and Severity of Ankylosing Spondylitis

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ABSTRACT. ***Objective.*** Unconfirmed reports describe association of ankylosing spondylitis (AS) with several candidate genes including *ANKH*. Cellular export of inorganic pyrophosphate is regulated by the ANK protein, and mutant mice (ank/ank), which have a premature stop codon in the 3' end of the *ank* gene, develop severe ankylosis. We tested the association between single-nucleotide polymorphisms (SNP) in these genes and susceptibility to AS in a population of patients with AS. We investigated the role of these genes in terms of functional (BASFI) and metrological (BASMI) measures, and the association with radiological severity (mSASSS).

Methods. Our study was conducted on 355 patients with AS and 95 ethnically matched healthy controls. AS was defined according to the modified New York criteria. Four SNP in *ANKH* (rs27356, rs26307, rs25957, and rs28006) were genotyped. Association analysis was performed using Cochrane-Armitage and linear regression tests for dichotomous and quantitative variables. Analyses of Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), BASFI, and mSASSS were controlled for sex and disease duration.

Results. None of the 4 markers showed significant single-locus disease associations ($p > 0.05$), suggesting that *ANKH* was not a major determinant of AS susceptibility in our population. No association was observed between these SNP and age at symptom onset, BASDAI, BASFI, BASMI, or mSASSS.

Conclusion. These results confirm data in white Europeans that *ANKH* is probably not a major determinant of susceptibility to AS. *ANKH* polymorphisms do not markedly influence AS disease severity, as measured by BASMI and mSASSS. (J Rheumatol First Release Nov 15 2011; doi:10.3899/jrheum.110681)

Key Indexing Terms:
ANKYLOSING SPONDYLITIS GENETIC PREDISPOSITION MORBIDITY

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Ankylosing spondylitis (AS) is a chronic inflammatory arthropathy, with an estimated prevalence of 0.1%–0.9% in white populations[1]. Genetic factors play a major role in the risk of developing AS[2,3], and influence several measures of disease severity, including the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Functional Index (BASFI)[4,5], and Bath AS radiographic index (BASRI)[6]. Studies have shown that sex influences susceptibility and disease severity. The prevalence of AS is 2.5 times higher in men than in women[7], and women have a later onset of the disease and less thoracic and lumbar spinal radiographic severity[8]. The pathophysiological mechanisms underlying these differences remain unclear. It is unlikely that the major genetic factors involved are X-linked because there is no linkage of AS susceptibility with X-chromosome markers[9].

The *ANKH* gene is of particular interest in AS, as mice with a loss-of-function mutation in the homologous gene, *ank*, develop severe ectopic mineralization and skeletal ankylosis resembling AS[10]. Humans with gain-of-expression mutations and polymorphisms in this gene develop calcium pyrophosphate chondrocalcinosis[11,12], whereas loss-of-function mutations cause excess hydroxyapatite deposition in Jackson's craniometaphyseal dysplasia disease[13,14]. A family has recently been described with a spondyloarthropathy with some similarities to AS due to homozygosity for a loss-of-function *ANKH* mutation[15]. An initial study of *ANKH* showed no association with susceptibility to AS[16], and no association has been identified with this gene in genomewide association studies in AS to date[17,18]. However, weak positive findings have been reported by some investigators[19], and it has been suggested that the association may be more strongly observed in women[20]. Further, no study has investigated the association of *ANKH* variants with radiographic or joint metrology indices. We sought to test the association between single-nucleotide polymorphisms (SNP) in *ANKH* and susceptibility to AS in a Portuguese population. Additionally we investigated the association of *ANKH* with functional (BASFI) and metrological (Bath Ankylosing Spondylitis Metrology Index; BASMI) measures, and for association with radiological severity (modified Stoke Ankylosing Spondylitis Spine Score; mSASSS).

MATERIALS AND METHODS

Subjects. The study was conducted on 355 unrelated patients with AS and 95 ethnically matched healthy controls, all of Portuguese ancestry. AS was defined according to the modified New York criteria[21]. Cases were recruited from hospital outpatient departments; controls were healthy Portuguese bone marrow donors. Our study was approved by the ethics board of the study centers involved, and written informed consent was obtained from the participating individuals.

Patients completed a questionnaire self-assessment of clinical features, including the BASDAI and the BASFI. Age at disease onset was defined as the age at symptom onset, and disease duration was defined as the period of time (years) after symptom onset. Metrology investigation was performed by 1 investigator (FPS) to obtain the BASMI score. Radiological evaluation was performed using the mSASSS; all radiographs were scored independently by 2 authors (FPS, AFM). Where there was discordance between the scores, they were reevaluated by both reviewers for a consensus score. Data on current therapy were collected.

Genotyping. Genomic DNA from cases and controls was prepared from peripheral blood lymphocytes using standard techniques. Samples were genotyped for *ANKH* allelic variants (rs27356, rs26307, rs25957, and rs28006) that had previously been associated with AS in either men or women[20]. Taqman® SNP assays (Applied Biosystems, Foster City, CA, USA) were used for genotyping, performed according to the manufacturer's protocols. Genotyping reactions were performed with an ABI 7900HT instrument, and the allele call by analysis of allelic discrimination plots with ABI SDS 2.3 software. Replicate known and negative genotype control samples were typed in each 96-well plate.

Statistical analysis. SNP genotype data were assessed for missing data and for Hardy-Weinberg equilibrium in controls. Individuals with > 10% missing-ness were excluded. Association analysis was performed using the Cochrane-Armitage test as implemented in the PLINK program (Harvard University, Cambridge, MA, USA; Website: http://pngu.mgh.harvard.edu/~purcell/plink/gplink.shtml). Association between SNP and the quantitative variables age of symptom onset, BASDAI, BASFI, BASMI, and mSASSS were tested by linear regression assuming an additive model using PLINK, taking into account sex and disease duration as covariates. Statistical power was tested using the Genetic Power Calculator[22].

RESULTS

The AS cohort population (n = 355) included 224 (63.1%) men and 131 (36.9%) women with a mean age of 45.4 (SD ± 13.2) years (range 20–79 yrs) and a mean disease duration of 19.1 (SD ± 12.6) years (range 0–60 yrs), of whom 82% were HLA-B27-positive. The therapies used were similar in all patients, with the exception of nonsteroidal antiinflammatory drugs, which were used in a greater percentage of women (92.6%) than men (80.4%). Thus differences in therapy between sexes are unlikely to be an explanation of observed differences in AS activity or severity. Epidemiological data of the cases are summarized in Table 1.

All genetic markers studied were in Hardy-Weinberg equilibrium in the control group, with missing-ness rates < 10%, and there were no observations of differential missing-ness in cases and controls ($p < 0.01$). The minor allele frequencies (MAF) of the 4 SNP are presented in Table 2.

None of the 4 studied markers showed significant single-locus disease associations ($p > 0.05$), in the whole group and in subanalysis by sex, suggesting that *ANKH* gene is not a major determinant of AS susceptibility in a Portuguese population (Table 2). In addition, no association was observed between these SNP and age of symptom onset, BASDAI, BASFI, BASMI, or mSASSS when considering the whole population (Table 3). The sample size in individual sex groups was too small for a worthwhile analysis.

The study had 80% power to detect association with AS ($p < 0.05$, assuming a population prevalence of 0.5%, with D' = 1) with an additive OR of 1.9, and to detect association with quantitative measures (BASDAI, BASFI, BASMI, mSASSS) contributing > 3% of the trait variance.

DISCUSSION

We analyzed 4 intronic markers previously described as asso-

Table 1. Characteristics of the Portuguese AS cases (n = 355). Except where indicated otherwise, values are the mean (standers deviation).

	Males	Females	p
No. (%)	224 (63)	131 (37)	NA
Age, yrs	45.49 (13.36)	44.88 (12.53)	NS
Age of symptom onset, yrs	25.73 (10.63)	27.20 (10.43)	NS
Disease duration, yrs	19.78 (12.17)	17.59 (12.86)	NS
BASDAI	3.75 (2.17)	4.89 (2.28)	< 0.01
BASFI	3.77 (2.63)	4.59 (2.69)	< 0.01
BASMI	4.26 (2.60)	3.56 (2.24)	< 0.05
mSASSS	26.97 (24.52)	10.16 (14.85)	< 0.05
Therapy (%)			
NSAID	182 (80.4)	122 (92.6)	NA
Corticosteroids	43 (19)	22 (16.5)	NA
DMARD	119 (52.6)	67 (50.4)	NA
Anti-TNF-α	55 (24.2)	27 (20.6)	NA

AS: ankylosing spondylitis; BASDAI: Bath AS Disease Activity Index; BASFI: Bath AS Functional Index; BASMI: Bath AS Metrology Index; mSASSS: modified Stoke Ankylosing Spondylitis Spine Score; NSAID: nonsteroidal antiinflammatory drugs; DMARD: disease-modifying antirheumatic drugs; NA: not applicable; NS: not significant; TNF: tumor necrosis factor.

ciated with AS in men (rs26307, rs27356) or women (rs28006, rs25957), in a study of 201 multiplex families[20]. In our study, involving unrelated patients with AS, we demonstrate that *ANKH* is not significantly associated with either susceptibility to AS or measures of its activity or severity, either in the whole group or in men or women separately. There are several possible explanations for the discrepancy of the results observed in the 2 studies: intrinsic differences between the 2 populations (North Americans vs Portuguese subjects) or differences in the patient populations (multiplex families vs unrelated individuals). Finally, both studies were underpowered to detect genes with small effects consistently, potentially leading to discrepancies between results. Despite the methodological differences (ethnicity, case ascertainment approaches, and *ANKH* marker variants analyzed), this investigation reinforces the results of another study in white Europeans[16], where no associations with disease susceptibility or phenotypic characteristics were seen. Our current study extends these previous observations, in that it is the first study to test *ANKH* associations with metrological (BASMI) and radiological (mSASSS) indices. Given the previous findings in mice and humans with loss-of-function *ANKH* mutants, we hypothesized that *ANKH* polymorphisms may contribute to spinal ossification in AS. This effect would be more easily detected by the potential influence on variables of the BASMI and mSASSS. Even considering these aspects, *ANKH* variants appeared to have no significant role in our population.

Several major ossification pathways have been identified that may play a central role in diseases characterized by bone formation, such as AS. They involve transforming growth factor-ß[23,24], bone morphogenetic proteins[25,26], and the wingless (Wnt) proteins[27,28]. Further studies investigating these pathways in larger datasets are indicated to identify genes influencing the severity and rate of ankylosis in AS.

Our results confirm previous data in white Britons that *ANKH* is not a major determinant of susceptibility to AS[16], and also demonstrate that *ANKH* variants do not have a major

Table 2. *ANKH* minor allele frequencies (MAF) in the Portuguese AS cohort.

		Males				Females			
NCBI SNP Reference	Minor Allele	MAF, Cases	MAF, Controls	p for trend	OR (95% CI)	MAF, Cases	MAF, Controls	p for trend	OR (95% CI)
rs26307	T	0.21	0.24	0.43	1.25 (0.82–1.92)	0.22	0.15	0.65	1.14 (0.73–1.78)
rs27356	C	0.22	0.22	0.29	1.35 (0.88–2.07)	0.21	0.17	0.61	1.16 (0.75–1.81)
rs28006	T	0.31	0.32	0.68	0.88 (0.59–1.31)	0.28	0.35	0.84	0.93 (0.62–1.41)
rs25957	C	0.31	0.34	0.70	0.89 (0.59–1.32)	0.28	0.35	0.81	0.92 (0.61–1.39)

AS: ankylosing spondylitis; NCBI: US National Center for Biotechnology Information; SNP: single-nucleotide polymorphism.

Table 3. Association between *ANKH* single-nucleotide polymorphisms and phenotypic characteristics of ankylosing spondylitis (AS).

	Age at Disease Onset	BASDAI	BASFI	BASMI	mSASSS*
rs26307	0.6311	0.7821	0.8728	0.1211	0.08/0.32/0.16
rs27356	0.6162	0.9569	0.9327	0.06895	0.14/0.28/0.22
rs28006	0.4899	0.442	0.09126	0.9675	0.3/0.43/0.73
rs25957	0.4577	0.4478	0.1072	0.577	0.34/0.4/0.88

* Men/women/total. BASDAI: Bath AS Disease Activity Index; BASFI: Bath AS Functional Index; BASMI: Bath AS Metrology Index; mSASSS: modified Stroke Ankylosing Spondylitis Spine Score.

influence on severity of AS (measured by BASDAI, BASFI, BASMI, or mSASSS) or age at disease onset.

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APPENDIX

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2.2.3.3. *TNFSF8*

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Systematic Candidate Gene Investigations in the SPA2 Locus (9q32) Show an Association Between *TNFSF8* and Susceptibility to Spondylarthritis

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Objective. **Our group previously identified a new susceptibility region linked to spondylarthritis (SpA) on chromosome 9q31–34. Fine mapping of this SPA2 locus allowed us to refine the peak of linkage to a 1.3-Mb interval. The objective of this study was to resequence most positional candidate genes lying in that region, to identify polymorphisms, and to examine their association with SpA.**

Methods. **Variants screening was performed in 30 independent patients with SpA from families with a high linkage score to the SPA2 locus and 30 control subjects. The coding regions, intron–exon boundaries, and 5′- and 3′-flanking regions of *ZNF618*, *A1L4R1_HUMAN* (AF495724), *AMBP*, *KIF12*, *ORM1*, *ORM2*, *C9ORF91*, *ENSESTG00000230601*, and *TNFSF8* were resequenced to identify polymorphisms. Selected variants were genotyped in an extended French cohort (442 patients and 268 control subjects overall). Replication was performed in a combined Belgian and Portuguese cohort (433 patients and 299 control subjects).**

Results. **Variants screening allowed us to identify 98 polymorphisms, 5 of which were selected for further studies, based on statistical significance. The rare intronic single-nucleotide polymorphism (SNP) rs3181357, located in *TNFSF8*, was significantly associated with SpA in the French and the replication cohorts (odds ratio [OR] 2.03, $P = 0.009$ and OR 2.26, $P = 0.0014$, respectively) and in the pooled analysis (OR 2.14, $P = 0.0001$).**

Conclusion. **Positional candidate gene screening in the SPA2 locus allowed us to identify and replicate an association between a rare SNP located in *TNFSF8* and SpA. This new finding appears to be independent of an association with a haplotype near *TNFSF15*, which we recently reported.**

Spondylarthritis (SpA) is one of the most common inflammatory rheumatic disorders, with an estimated prevalence of 0.3% in adults living in western Europe (1). It is characterized by axial and/or peripheral arthritis frequently associated with extraarticular manifestations, such as psoriasis, acute anterior uveitis, and inflammatory bowel disease (IBD, i.e., ulcerative colitis or Crohn's disease) (2). Diagnostic entities that comprise SpA are ankylosing spondylitis (AS), characterized by predominant axial skeletal involvement and advanced radiographic sacroiliitis; reactive arthritis, following a triggering infection; a subset of psoriatic arthritis; arthri-

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tis associated with IBD; and undifferentiated SpA. All of these subsets share genetic predisposition, as shown by their striking tendency toward familial aggregation of cases (3) and also by their strong association with HLA–B27 (4). Extensive investigations in a large panel of families with multiple cases of SpA led us to conclude that all of the subtypes should be studied together for the purpose of genetic investigations (5–7).

Familial clustering and twin studies have demonstrated that the predisposition to SpA was not exclusively related to HLA–B27. Notably, a recent study estimated the weight of the non–major histocompatibility complex (non-MHC) genetic component to be half that of the whole genetic predisposition to the disease (8). Besides, several linkage and association studies have shown that susceptibility genes are expected in SpA, arising from outside the MHC (9).

A genome-wide screen performed by our team in multiple-case SpA families allowed us to identify a highly significantly linked region on chromosome 9q31–34, with a maximum nonparametric linkage (NPL) score of 4.87 ($P = 2 \times 10^{-5}$) (10). This locus spanning 23.95 cM (17.44 Mb) was overlapping with one of those identified by a group of investigators from Oxford in a linkage study of AS sibling pairs (11). Thus, we named it SPA2. Interestingly, it is 1 of 3 genomic regions paralogous to the MHC, which is the major SpA susceptibility locus (12,13). It is also syntenic to the *Pgis*2 susceptibility locus mapped in a murine model of SpA (14).

In our genome-wide screen, the peak of linkage in the SPA2 region was centered by the microsatellite marker D9S1776, mapped at 118 Mb from the p telomere (www.ensembl.org). Further linkage studies using a denser set of microsatellite markers in an extended collection of families allowed us to refine this peak to an interval spanning 1.28 Mb in 9q32, between markers D9S279 and D9S1855, which is located 440 kb centromeric to D9S1776 (15). As yet, 11 genes have been mapped to this refined target region (www.ensembl.org). In the present study, we investigated whether 8 of these genes are involved in susceptibility to SpA. We also studied *TNFSF8*, an additional major candidate gene located between D9S1855 and D9S1776.

It is noteworthy that we have already extensively studied *TNFSF15* and *TNC*, 2 other positional and functional candidate genes located in the interval D9S1855–D9S1776, and the negative results of those studies were reported elsewhere (15,16). However, using a systematic linkage disequilibrium mapping strategy, we recently identified a 6–single-nucleotide polymorphism (SNP) haplotype near *TNFSF15* that is significantly associated with SpA (15).

In the current study, we first performed direct resequencing of the coding regions, intron–exon boundaries, and 5′- and 3′-flanking regions of the selected candidate genes, to compare the variability of those genes in SpA patients from linked families with that in controls. SNPs showing suggestive association were then genotyped in larger panels of patients and controls.

PATIENTS AND METHODS

Study population. French SpA patients and control subjects were recruited through the Groupe Français d'Etude Génétique des Spondylarthropathies. Subjects in the Belgian case–control series were recruited through the Rheumatology outpatient clinics of Ghent and Leuven University Hospitals. The Portuguese case–control collection was recruited through the Rheumatology Division of Lisboa University Hospital de Egas Moniz. The project was approved by relevant local ethics committees, and informed consent was obtained from each participant. SpA patients from the French and Belgian collections satisfied the classification criteria proposed by Amor et al (17) and/or the European Spondylarthropathy Study Group (18), as previously described (5). The characteristics of those patients are shown in detail in Table 1. Among the 150 patients from the Portuguese collection, 91 were men, and 59 were women (mean ± SD age 46.7 ± 13.4 years); all of the patients fulfilled the modified New York criteria for AS (19). Control subjects were matched to the patients by ethnicity.

In the discovery part of the study, 2 independent French case–control samples were consecutively analyzed in a 2-stage strategy. Variants screening was performed on a panel of 15 independent patients with SpA and 15 control subjects. DNA fragments containing polymorphisms suggestively associated with the disease were then resequenced in another set of 15 patients with SpA and 15 control subjects. The 30 patients included in this first part of the study were selected from French families with the highest NPL scores in the SPA2 region (NPL ≥1.34). The extension study was performed in an additional French panel of 412 patients with SpA and 238 control subjects.

A replication study of SNP rs3181357 was performed in a combined Belgian and Portuguese panel consisting of 433 independent patients and 299 healthy control subjects; the study had 60% power to replicate an association ($P = 0.05$) with odds ratios (ORs) of >1.95 considering a minor allele frequency of 0.03 for this marker.

Candidate genes. Eight candidate genes in the SPA2 region were selected from a list of 11 genes identified between microsatellite markers D9S279 and D9S1855. We also studied *TNFSF8*, which is located near D9S1855, between this marker and D9S1776. These genes were identified using the public databases Ensembl (www.ensembl.org) and UCSC (http://genome.ucsc.edu/cgi-bin/hgGateway). Information about these genes is shown in Table 2.

Variants screening. The coding regions, intron–exon boundaries, and 5′- and 3′-flanking regions of the genes were

Table 1. Characteristics of the French and Belgian patients with spondylarthritis included in the study*

Characteristic	French patients		Belgian patients, replication study (n = 283)
	Variants screening (n = 30)	Extension study (n = 412)	
Age, mean ± SD years	47 ± 3	51 ± 0.65	49 ± 0.89
Age at onset, mean ± SD years	23 ± 3	26 ± 0.51	28 ± 0.83
No. of men/no. of women	16/14	275/137	185/98
HLA–B27 positive, no. (%)	100	80	76
Axial manifestations, %			
Back/buttocks pain	100	96	90
Sacroiliitis†	87	48	79
Peripheral manifestations, %			
Peripheral arthritis	43	39	54
Peripheral enthesitis	60	79	36
Extraarticular manifestations, %			
Uveitis	40	25	27
Psoriasis	20	27	5
Inflammatory bowel disease‡	17	10	7
Spondylarthritis subtype, %			
Ankylosing spondylitis	87	48	79
Undifferentiated	10	36	20
Psoriatic arthritis	3	14	0
Arthritis associated with IBD	0	3	1.5

* The registered manifestations correspond to those that were present at the time of examination or those that were retrieved from the patient's medical history. IBD = inflammatory bowel disease.
† Radiographic, at least grade II bilaterally or grade III unilaterally.
‡ Crohn's disease or ulcerative colitis.

amplified using Thermo-Start DNA Polymerase Mastermix (Abgene). Polymerase chain reactions (PCRs) were performed on 25–50 ng of genomic DNA according to the manufacturer's recommendations. Before sequencing, PCR products were purified by means of a modified polyethylene glycol precipitation protocol. Dried products were then dissolved in 5 m*M* Tris, pH 9.0. Approximately 10–30 ng of purified products was sequenced using BigDye Terminator chemistry on ABI 3730xl capillary sequencers (Applied Biosystems). Primers design, PCR, and sequencing reactions were performed by Eurofins MWG. The PCR and resequencing primer sequences are available from the corresponding author. DNA sequences were analyzed using SeqScape software (Applied Biosystems).

SNP genotyping. Five SNPs were genotyped in the extension French case–control panel. Genotyping was performed using a high-throughput production-scale 48-plex assay (Applied Biosystems) or melting curve analyses (LightCycler System; Roche). Automatic allele calling was done using GeneMapper software version 4.0 (Applied Biosystems) with the rules clustering algorithm.

Polymorphism annotation and statistical analysis. Variants were positioned and annotated using the Ensembl

Table 2. Description of the 9 candidate genes selected for the study*

Gene name†	Ensembl location, bp	HUGO gene name‡	Transcription strand§	No. of exons¶	Alternative splicing
ZNF618	116,638,562–116,818,871	HGNC:29416	+1	16	Yes
A1L4R1_HUMAN (AF495724)	116,821,884–116,822,673	–	+1	1	No
AMBP	116,822,410–116,840,752	HGNC:453	−1	10	No
KIF12	116,853,920–116,861,556	HGNC:21495	−1	19	Yes
ORM1	117,085,303–117,088,757	HGNC:8498	+1	6	No
ORM2	117,092,074–117,095,534	HGNC:8499	+1	6	No
C9ORF91	117,373,706–117,408,696	HGNC:24513	+1	10	No
ENSESTG00000230601	117,429,626–116,445,101	–	−1	5	No
TNFSF8	117,665,124–117,692,770	HGNC:11938	−1	5	No

* Two candidate genes located in the same interval, *TNFSF15* and *TNC* (HGNC:11931 and HGNC:5318, respectively) have been studied elsewhere (15,16). One remaining positional candidate gene, *COL27A1* (HGNC:22986), containing 61 exons has not been studied.
† According to the Ensembl nomenclature.
‡ According to the HUGO nomenclature (http://www.genenames.org/).
§ +1 = gene transcribed in forward strand; −1 = gene transcribed in reverse strand.
¶ All known transcribed exons (coding or noncoding).

Table 3. Association results for all SNPs with a suggestive *P* value (by Cochran-Armitage trend test) in the first step of the study*

SNP name	Gene name	Ensembl location, bp	A1†	A1 frequency		A2‡	OR§	Cochran-Armitage statistic value	Empirical *P*, unadjusted¶	Empirical *P*, by permutation method	Gene location
				Patients	Controls						
Unknown (1)	*ZNF618*	116,795,061	T	0.18	0.07	C	3.14	4.36	0.037	0.079	Intronic
rs10118282	*ZNF618*	116,800,924	A	0.20	0.07	G	3.38	5.12	0.024	0.042	Intronic
rs12378906	*ZNF618*	116,810,204	A	0.18	0.07	G	3.14	4.36	0.037	0.079	Coding (synonymous SNP)
Unknown (2)	*KIF12*	116,859,852	A	0.10	0.30	G	0.26	3.91	0.048	0.055	Intronic
rs1107080 (1)	*ORM1*	117,085,186	G	0.00	0.17	C	0.00	4.05	0.044	0.050	5′ upstream
rs2787338	*ORM1*	117,085,613	C	0.50	0.37	G	1.73	4.62	0.032	0.050	Intronic
Unknown (5)	*ORM1*	117,086,241	A	0.00	0.17	G	0.00	4.05	0.044	0.050	Intronic
rs10982154	*ORM1*	117,087,212	A	0.00	0.13	T	0.00	4.62	0.032	0.050	Intronic
rs10982155	*ORM1*	117,087,254	T	0.00	0.13	C	0.00	4.62	0.032	0.050	Intronic
ENSSNP2918740	*ORM2*	117,091,896	G	0.00	0.13	C	0.00	4.62	0.032	0.050	5′ upstream
rs1687417	*ORM2*	117,095,146	C	0.03	0.20	T	0.14	4.66	0.031	0.039	Intronic
rs7036717	*C9ORF91*	117,386,348	A	0.43	0.13	G	4.97	5.27	0.022	0.022	Intronic
rs7031094	*C9ORF91*	117,390,300	C	0.37	0.15	A	3.28	6.26	0.012	0.023	Intronic
rs751780	*C9ORF91*	117,395,881	C	0.32	0.12	A	3.51	5.39	0.020	0.038	Intronic
rs3810935	*C9ORF91*	117,396,237	T	0.27	0.10	C	3.27	4.18	0.041	0.064	Intronic
rs3181357	*TNFSF8*	117,692,344	T	0.05	0.00	C	NA	3.16	0.076	0.240	Intronic

* Single-nucleotide polymorphisms (SNPs) shown in boldface were tested on a sample of 30 patients and 30 controls; all others were tested on an initial sample of 15 patients and 15 controls. NA = not applicable.
† Minor allele, based on the whole sample.
‡ Major allele.
§ Odds ratio (OR), (A1 in patients/A2 in patients)/(A1 in controls/A2 in controls).
¶ For the Cochran-Armitage trend test.

database (www.ensembl.org). All identified SNPs were tested for departure from Hardy-Weinberg equilibrium, using the exact Hardy-Weinberg test, which is more accurate than the asymptotic test for rare genotypes (20). Association between single variants and the disease was assessed by the Cochran-Armitage trend test using PLINK software (21). Correction for multiple testing was performed, using the permutation method in the discovery step of the study and Bonferroni correction for 5 tests in the extension step. Homogeneity of allelic distribution between cohorts for rs3181357 was assessed using StatXact 8 software (Cytel). Conditioning analysis was performed using UnPhased 3.1.4 (www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/). Pairwise correlation between the 6 previously reported haplotype-tagging SNPs situated near *TNFSF15* (15) and rs3181357 was studied using the Haploview program (www.broad.mit.edu/mpg/haploview/).

RESULTS

We selected 9 genes in the SPA2 region, *ZNF618*, *A1L4R1_HUMAN* (AF495724), *AMBP*, *KIF12*, *ORM1*, *ORM2*, *C9ORF91*, *ENSESTG00000230601*, and *TNFSF8*, to be studied for their putative implication in SpA susceptibility. Overall, we directly resequenced 41,708 bp, 9,591 of which were coding. All exons were successfully resequenced. A total of 98 SNPs were identified by this investigation (1 polymorphism every 425 bp), which included 7 nonsynonymous SNPs (additional information is available from the corresponding author). The genotyping success rate for individual SNPs varied between 93.3% and 100%. Each gene

Table 4. Association results for the 5 SNPs genotyped in the extension study performed in a French cohort of 442 patients with spondylarthritis and 268 control subjects*

SNP name	Gene	Ensembl location, bp	A1	A2	A1 frequency		OR (95% CI)	Unadjusted *P*†
					Patients	Controls		
rs10118282	*ZNF618*	116,800,924	A	G	0.125	0.114	1.11 (0.73–1.68)	0.619
rs7031094	*C9ORF91*	117,390,300	C	A	0.312	0.289	1.12 (0.88–1.41)	0.888
rs751780	*C9ORF91*	117,395,881	C	A	0.251	0.238	1.06 (0.83–1.37)	0.601
rs3810935	*C9ORF91*	117,396,237	T	C	0.237	0.215	1.13 (0.86–1.48)	0.351
rs3181357	*TNFSF8*	117,692,344	T	C	0.052	0.026	2.03 (1.10–3.74)	0.009

* A1 is the minor allele, and A2 is the major allele. SNPs = single-nucleotide polymorphisms; OR = odds ratio; 95% CI = 95% confidence interval.
† Value for the Cochran-Armitage test, after multiplicity correction.

Table 5. Association results for SNP rs3181357 (*TNFSF8*) in the Belgian and Portuguese cohorts*

Cohort	A1 frequency	OR (95% CI)	P†
Belgian			
Patients (n = 283)	0.086	2.47 (1.26–4.83)	0.006
Controls (n = 149)	0.036		
Portuguese			
Patients (n = 150)	0.046	1.58 (0.67–3.71)	0.287
Controls (n = 150)	0.03		

* A1 (T) is the minor allele (based on the whole sample). OR = odds ratio; 95% CI = 95% confidence interval.
† Value for the Cochran-Armitage test.

except *C9ORF91* had at least 1 coding SNP, which was synonymous or not synonymous. The average numbers of polymorphisms/kb of DNA were 1.56 in the coding regions and 2.58 in the noncoding regions. None of the genotype distributions in control subjects differed significantly from those expected based on Hardy-Weinberg equilibrium (data not shown).

Fifteen (15.3%) of 98 variants located in 5 genes reached unadjusted significance for association ($P < 0.05$) (Table 3). Five of these SNPs attained significance after correction for multiple testing by the permutation method. The OR was >1 for 2 of these 5 SNPs (1 each in *ZNF618* and *C9ORF91*), meaning that the rare allele was more frequent in patients than in control subjects. Finally, 1 SNP located in *TNFSF8* did not reach the significance threshold; however, the rare allele T was identified only in patients.

We further genotyped the 5 most suggestively associated SNPs resulting from the foregoing discovery study (rs10118282, rs7031094, rs751780, rs3810935, and rs3181357) in an additional independent French sample and performed final analysis on the whole French data set consisting of 442 patients and 268 control subjects (Table 4). One single SNP, rs3181357, located in *TNFSF8*, yielded a significant *P* value for association, which persisted after multiplicity correction (OR 2.03, 95% confidence interval [95% CI] 1.10–3.74, $P = 0.009$) (Table 4).

In order to replicate this finding, rs3181357 was further genotyped in another combined case–control cohort from Belgium (283 patients and 149 control subjects) and Portugal (150 patients and 150 control subjects) (Table 5). The allelic frequency of rs318135 appeared to be homogeneous across all cohorts, allowing us to perform combined stratified analyses (Table 6). There was a significant association in the replication cohort (OR 2.26, 95% CI 1.35–3.79, $P = 0.0014$) (Table 6). The pooled analysis between all samples resulted in a highly significant association of the rare allele of rs3181357 with SpA (OR 2.14, $P = 0.0001$) (Table 6).

Having previously reported an association between a 6-SNP haplotype situated near *TNFSF15* and SpA (15), we examined whether the association with rs3181357 was independent of the former, using the French data set genotyped in both studies. Testing for association between rs3181357 and SpA, conditioning on the previously described haplotype (15), showed their independence. Furthermore, there was no linkage disequilibrium between any of the 6 haplotype-tagging SNPs and rs3181357 ($r^2 \leq 0.006$ for all pairwise correlation tests).

DISCUSSION

A genome-wide linkage study formerly performed by our team on a large panel of pedigrees with multiple cases of SpA allowed us to identify a new region

Table 6. Association results for rs3181357 (*TNFSF8*) in the combined replication cohort and pooled analysis*

Cohort	A1 frequency	*P* for homogeneity†	OR (95% CI)	P‡
Combined Belgian and Portuguese				
Patients (n = 433)	0.072	0.58	2.26 (1.35–3.79)	0.0014
Controls (n = 299)	0.033			
Pooled (French, Belgian, and Portuguese)				
Patients (n = 875)	0.062	0.83	2.14 (1.15–3.17)	0.0001
Controls (n = 567)	0.03			

* A1 (T) is the minor allele (based on the whole sample). OR = odds ratio; 95% CI = 95% confidence interval.
† The test for homogeneity was performed with StatXact 8 software (Cytel).
‡ Value for the Cochran-Armitage test.

of susceptibility to SpA on chromosome 9q31–34, that we named SPA2 because it was the first replicated non-MHC locus linked to SpA (10). This locus should contain major predisposition factor(s), accounting for 20–25% of the non-MHC genetic susceptibility to SpA (2). It contains ~85 genes and several predicted coding sequences. Most interestingly, it is also one of the 3 regions paralogous to the MHC, the major susceptibility locus to SpA, which may contain additional susceptibility genes besides HLA–B27 (4). Thus, one or more gene(s) in SPA2, possibly having paralogous counterparts in the MHC, could be implicated in the genetic susceptibility to SpA.

Since our initial discovery, we have refined the peak of linkage to a 1.28-Mb interval containing a set of 11 genes, which was accessible for a systematic study (15). One of our strategies was to explore whether polymorphisms in the coding and neighboring regions of the positional candidate genes were associated with disease. In the current study, we selected 8 of these 11 genes to be studied in priority. Some of them, such as *ORM1*, *ORM2*, and *AMBP*, were very attractive functional candidate genes, because they code for acute-phase proteins regulating inflammation. Besides, *KIF12* is one of the genes in this region having a paralogous counterpart in the MHC locus, i.e., *KIFC1*. Given that linkage studies have rather limited precision in complex disorders such as SpA, we added to our list *TNFSF8*, one of the major functional candidate genes situated in the vicinity of the above-defined interval and with a paralogous counterpart in the MHC.

We first resequenced the 78 exons, the intron–exon boundaries, and the 5′- and 3′-flanking regions of the 9 genes, in a screening sample of 15 patients and 15 control subjects. This allowed us to identify 98 SNPs, 12 of which were not annotated in public databases. Regions containing SNPs with suggestive association in this first data set were then resequenced in a second sample of 15 patients and 15 control subjects. This discovery step was performed in independent patients from families with the highest linkage scores to the SPA2 locus, in order to maximize our chance to identify rare causal variant(s). Nevertheless, because the number of sequenced patients and control subjects was rather low, one cannot entirely exclude that there could be more rare variants that were missed. Overall, resequencing data allowed us to identify 5 SNPs within *ZNF618*, *C9ORF91*, and *TNFSF8* that were of potential interest in terms of the predisposition to SpA.

In a second stage, we investigated these 5 SNPs in an additional French sample and performed final analysis on the whole French data set of 442 patients and 268 control subjects. No significant association was observed with the 4 SNPs having a minor allele frequency of >0.1. This panel had 80–99% power to detect such an association ($P = 0.05$) with an OR of >1.9, but less than 50% power with an OR of <1.2. Thus, it cannot be ruled out that some of these SNPs could play a weak role in disease predisposition, but it is unlikely that any of them would account for the strong linkage signal observed at the SPA2 locus. The only significant association was with SNP rs3181357. Because we replicated this association in an independent combined Belgian and Portuguese cohort, it is likely to be a true positive finding. It is noteworthy that those cohorts were combined to maximize the power of replicating our finding at a significant level. This SNP is intronic, positioned downstream of the first exon on *TNFSF8*. This polymorphism by itself could affect the expression and/or splicing of *TNFSF8*.

TNFSF8, which has a paralogous counterpart in the MHC, is a very relevant functional candidate gene in SpA. Indeed, it codes for CD30L (CD153), a tumor necrosis factor (TNF) superfamily ligand expressed on activated CD4+ T cells, antigen-presenting cells, and neutrophils, which interacts with CD30 on effector or memory T helper cells. It is thought to play a role in inflammatory conditions related to SpA, such as IBD (22), and it was shown that CD30L/CD30 signaling plays a critical role in Th17 cell differentiation in vitro and in vivo (23). Interestingly, there is evidence that Th17 cells could be involved in the pathogenesis of SpA (24,25). Furthermore, several other non-MHC genetic factors recently shown to be associated with SpA are involved both in Th17 cell differentiation and in predisposition to IBD (26). It is worth mentioning that this is the case for *TNFSF15*, the second member of the TNF superfamily of genes residing in the SPA2 region next to *TNFSF8* (9,27).

We recently completed systematic linkage disequilibrium mapping of the whole SPA2 region by using a dense set of tag SNPs, which allowed us to identify an association of SpA with a 6-SNP haplotype spanning 40 kb and located between *C9ORF91* and *TNFSF15*. This association overlaps with that described between *TNFSF15* and Crohn's disease (27). The corresponding linkage disequilibrium block is situated only 140 kb centromeric to rs3181357 (15). Hence, the *TNFSF8* SNP described herein as associated with SpA could be directly implicated in SpA predisposition, alone or in combination with other polymorphisms situated in the same region.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Breban had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Zinovieva, Kadi, Letourneur, Izac, Vigier, Said-Nahal, Chiocchia, Breban.

Acquisition of data. Zinovieva, Kadi, Letourneur, Izac, Vigier, Said-Nahal, Elewaut, de Vlam, Pimentel-Santos.

Analysis and interpretation of data. Zinovieva, Kadi, Letourneur, Cagnard, Izac, Vigier, Elewaut, Chiocchia, Breban.

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2.3. GENE EXPRESSION STUDIES

2.3.1. Brief review on Microarray technology

Recent advances in molecular biology, in particular, the completion of the genome human sequence, the improvement in computational tools and the rapid access to large databases, allow an integrated understanding of biological systems, through "omic" approaches. The main challenge, however, is to extract relevant knowledge from the huge amount of data provided by these technologies for the development of biomarkers for diagnosis, prognosis, therapy monitoring and both prediction and monitoring of treatment response. Such technological advances represent the beginning of patient-specific personalized medicine [Kandpal *et al.*, 2009].

In contrast to traditional DNA-based diagnostic tests that largely focus on single genes associated with rare conditions, microarray-based genotyping and expression assays are ideal for the study of diseases with underlying complex genetic causes [Li *et al.*, 2008]. Microarray gene expression technology can be used for the detection and quantification of differentially expressed genes. Its ability to study expression of several thousand genes or even all of the genes of the entire genome in a single experiment has changed biomedical research. Gene-expression profiling confers a "snapshot" of cellular activity providing information on the mechanisms mediating stress responses of human cells [Belcher *et al.*, 2000; Guillemin *et al.*, 2002], identification of signaling cascades [Shaffer *et al.*, 2000; Diehn *et al.*, 2002], disease changes, or mechanisms underlying therapy responses [Raetz & Moos, 2004]. It represents an advance to the traditional molecular genomic techniques that have been previously applied in

broad clinical research fields as cancer, infection diseases, metabolic diseases, genetics and more recently, in rheumatic diseases.

2.3.1.1. Microarray fundamentals

Gene expression techniques, based on measuring mRNA levels, have greatly evolved since the development of the Northern blot, in 1975 [Southern, 1975] to microarrays, in the mid 1990s [Shalon *et al.*, 1996]. From a single labeled mRNA (probe), hybridized on a membrane (Northern Blot), to multiple probes hybridized on a membrane (macroarrays) or on glass (microarrays), the improvement was tremendous. Today several platforms, with pre-designed and custom arrays are available in the market [Hardiman, 2004] from commercial suppliers including Affymetrix, Agilent and Illumina. Table 1 summarizes similarities and differences between the most widely used platforms.

Table 1: Microarray platforms comparison.

	Platforms		
	Affymetrix	**Agilent**	**Illumina**
Array format	25-mer	60-mer	50-mer
Starting RNA requirement	5µg total RNA	**Fluorescent Direct Label Kit (cDNA labeling):** 10µg total RNA, or 200ng polyA+ RNA **Low input RNA Fluorescent Linear Amplification kit (Amplified cDNA labeling):** 50ng total RNA **Low input RNA Fluorescent Linear Amplification kit (Amplified cRNA labeling):** 50ng total RNA	50-500ng total RNA
Hybridization time	16h	**Fluorescent Direct Label Kit:** 3-4 hours **Low input RNA Fluorescent Linear Amplification kit Amplified cDNA labeling**: 10 hours **Amplified cRNA labeling:** 6 hours	16h
Hybridization temperature	45ºC	60ºC	55ºC
Detection method	Streptavidin-phycoerythrin	Cyanine 3 (Cy3) and cyanine 5 (Cy5) Fluorescent labeling	Streptavidin-Cy3
Advantages	Reproducibility; Full genome coverage; mature platform; customization More probes per gene	Reproducibility; content; mature platform; sensitivity; customization	Reproducibility; Full genome coverage Sensitivity Low background Mature platform Low cost/sample Low starting material required
Disadvantages	Short oligonucleotides-less sensitive High cost/sample	Two-color dye bias and ozone-related degradation	Currently only available for human, rat and mouse studies Fewer probes per gene, not so sensitive to detect splice variants.

Despite minor differences between platforms, the basic steps involved in a microarrays experiment are similar (Fig. 1) [Repsilber *et al.*, 2005]. Key points in undertaking an expression profiling study are;

a) Establish your research question which will influence
b) Selection of the tissue/cell most relevant to the question and the selection of the control group
c) Total mRNA is extracted from the chosen tissue/cell, and reverse transcribed generating cDNA which is labeled with radioactive or fluorescent markers
d) Labeled transcripts are hybridized onto the microarray
e) Bound probes are detected and quantified by imaging tools and every gene/probe assigned a signal intensity
f) Signals are corrected for common bias i.e. normalized. For each mRNA, the signal intensity difference between the disease and the control sample correlates to the change in gene expression (genes up- or down-regulated) that might be associated with the studied condition. Several methods have been implemented to reduce variability in DNA microarray experiments [Workman *et al.*, 2002]. A critical step in the whole procedure is an appropriate analysis of the large volumes of data generated using sophisticated software. Bioconductor (www.bioconductor.org) or BRB ArrayTools [Simon *et al.*, 2007], examples of bioinformatics' platforms, provide tools for analysis and comprehension of genomic data.
g) Candidate genes are validated through another technology. Usually quantitative reverse-transcription PCR (qPCR) is the preferred method
h) Data is integrated and applied to the initial question.

2.3.1.2. Microarray challenges and concerns

Large-scale gene expression analysis, is in fact, a flourishing technology with potential applications in several fields of Biology and Medicine as indicated by the large number of peer-reviewed articles (n=35502) containing the words “gene” and “microarray” found in “Pubmed” up to June 2011.

Microarray profiling of gene expression is a powerful tool for discovery, but the ability to manage and compare the resulting data can be problematic. Biological, experimental, and technical variations between studies of the same phenotype/phenomena create substantial differences in results. Some of these issues will be discussed in detail.

a) The success of the microarray experience greatly depends on whether the hypothesis and rationale have been appropriately formulated through a clearly delineated question. It influences the study design as a whole, from sample collection, to experimental design and finally, the strategies for data analysis [Smith & Rosa, 2007].

b) While most of the early studies used primary tissues involved in the disease, such as tumor biopsies, more recently a number of gene expression profiling studies have focused on peripheral blood to identify systemic markers of disease. However, gene expression patterns in peripheral blood cells greatly depend on inter-individual variations and technical aspects such as blood sampling techniques, cell and RNA isolation as well as storage temperature or delays in processing. However although significant inter-individual variations on gene expression patterns in peripheral blood

cells can be seen, these differences are often much less than the differences between blood samples from healthy donors and from patients. These observations and the accessibility of peripheral blood, strongly suggests that gene expression analysis of peripheral blood is probably the best source for the assessment of systemic differences or changes in gene expression associated with disease or drug response [Debey *et al.*, 2004].

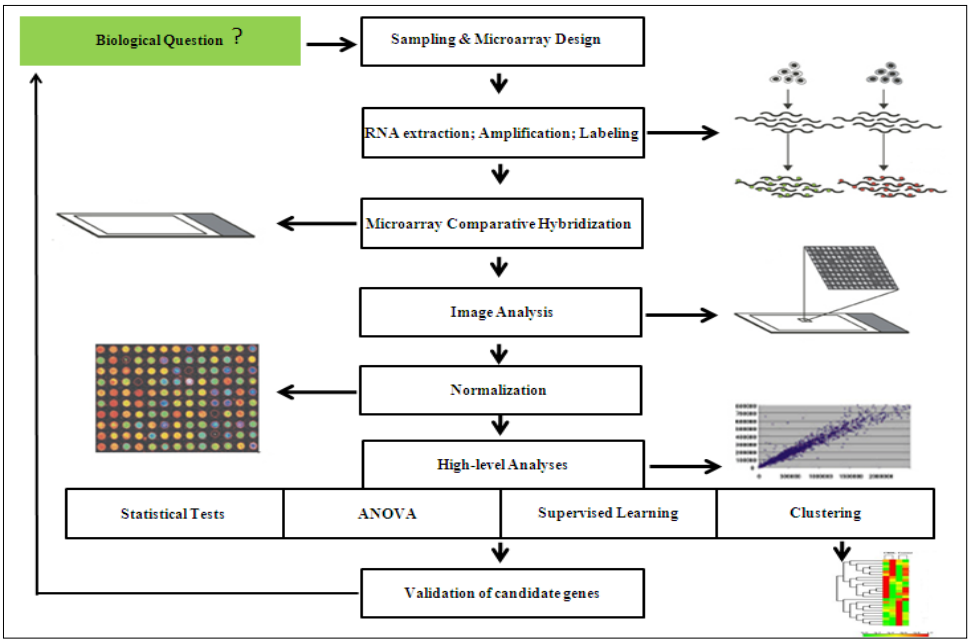

Figure 1: Design, experimental and data analysis steps in a typical microarray gene expression experiment. (Adapted from Repsilber *et al.*, 2005).

c) Appropriate experimental design is another critical step for the success of a microarray experiment. It's important to control and exclude as many biases as possible [Ransohoff, 2007]. Integrity and purity of RNA extracted, cDNA labeling and hybridization procedures may affect reproducibility, and thus these steps need to be standardized and optimized. However, several key issues regarding appropriate

replication remains in discussion: the minimum sample size, the necessity of running multiple arrays with the same samples or the potential benefits and risks associated with pooling samples [Smith & Rosa, 2007]. Increasing the sample size will lower the false discovery and false negative rates but it represents an expensive option [Pawitan *et al.*, 2005]. Given the well-established reproducibility of commercially available platforms, technical replication is not required currently. Finally, pooling samples can reduce the variation between arrays but potential outliers may get masked or may compromise the entire pool [Smith & Rosa, 2007]. To guaranty an improvement of data quality, replication studies in independent patient series must be performed, but these analyses are often lacking [Ionnidis *et al.*, 2009].

d) Data analysis currently represents a major challenge for researchers. A closer look at the literature reveals many conflicting results. A consensus regarding strategies in data analysis is required. In the last years some papers have reviewed in detail how to analyze typical microarray data experiments [Allison *et al.*, 2006; Reimers, 2010], to interpret them [Michiels *et al.*, 2007] and to report the results [Dupuy & Simon, 2007]. The multidimensionality of microarrays and possible solutions to deal with this issue is well discussed in a recent review [Michiels *et al.*, 2011].

e) Confirmation and validation studies are another crucial step. For confirmation studies the initial results must be reproduced using another assay technology, usually qPCR. Validation studies require an independent study in a new sample cohort to confirm that the gene signatures defined previously replicate satisfactorily in a similar clinical setting. It may be performed by the same research team or ideally by others.

These additional steps reduce false positives and the potential for biases [Michiels *et al.*, 2007, 2011].

f) Establishing a consensus to optimize each step of the procedure would therefore generate more reproducibility in results from different studies. Evidence-based guidelines to perform meta-analysis of array data are in progress [Ramasamy *et al.*, 2008] but establishing consensus in experimental design and protocols is still the most likely method to minimize variation. Clinical trials to confirm the gene signature's clinical utility on diagnosis and treatment decisions are mandatory, after the identification of reliable biomarkers.

The data from our research work are presented below.

2.3.2. RNA microarray analysis

Pimentel-Santos FM, Ligeiro D, Matos M, Mourão AF, Costa J, Santos H, Barcelos A, Godinho F, Pinto P, Cruz M, Fonseca JE, Guedes-Pinto H, Branco JC, Brown MA, Thomas GP. **Whole blood transcriptional profiling in ankylosing spondylitis identifies novel candidate genes that might contribute to the inflammatory and tissue-destructive disease aspects.** *Arthritis Res Ther*. 2011 Apr 7; 13(2): R57.

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RESEARCH ARTICLE Open Access

Whole blood transcriptional profiling in ankylosing spondylitis identifies novel candidate genes that might contribute to the inflammatory and tissue-destructive disease aspects

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Abstract

Introduction: A number of genetic-association studies have identified genes contributing to ankylosing spondylitis (AS) susceptibility but such approaches provide little information as to the gene activity changes occurring during the disease process. Transcriptional profiling generates a 'snapshot' of the sampled cells' activity and thus can provide insights into the molecular processes driving the disease process. We undertook a whole-genome microarray approach to identify candidate genes associated with AS and validated these gene-expression changes in a larger sample cohort.

Methods: A total of 18 active AS patients, classified according to the New York criteria, and 18 gender- and age-matched controls were profiled using Illumina HT-12 whole-genome expression BeadChips which carry cDNAs for 48,000 genes and transcripts. Class comparison analysis identified a number of differentially expressed candidate genes. These candidate genes were then validated in a larger cohort using qPCR-based TaqMan low density arrays (TLDAs).

Results: A total of 239 probes corresponding to 221 genes were identified as being significantly different between patients and controls with a *P*-value <0.0005 (80% confidence level of false discovery rate). Forty-seven genes were then selected for validation studies, using the TLDAs. Thirteen of these genes were validated in the second patient cohort with 12 downregulated 1.3- to 2-fold and only 1 upregulated (1.6-fold). Among a number of identified genes with well-documented inflammatory roles we also validated genes that might be of great interest to the understanding of AS progression such as *SPOCK2* (osteonectin) and *EP300*, which modulate cartilage and bone metabolism.

Conclusions: We have validated a gene expression signature for AS from whole blood and identified strong candidate genes that may play roles in both the inflammatory and joint destruction aspects of the disease.

Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease characterised by inflammation that leads to bone resorption and bone formation, ultimately resulting in progressive ankylosis [1]. Although the aetiopathogenesis of AS is not yet clearly defined, both susceptibility to and severity of this disease are highly heritable. The major gene association is with the MHC I gene *HLA-B27* with 95% of patients positive for this gene [2-4]. However, only approximately 5% of *HLA-B27* carriers suffer from AS, meaning other genes are involved in disease susceptibility. In fact, twin and family studies have suggested that *HLA-B27* accounts for less than 40% of the overall risk for AS [2,4]. In recent years genetic-association studies have identified several new genes in association with AS. Some of these genes

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appear specific for AS, whereas others have pleiotropic associations [5,6]. Nevertheless, the mechanism by which *HLA-B27* and other more recently identified genetic factors involved in AS susceptibility, lead to disease remains uncertain.

Genetic studies provide little information as to the gene activity changes occurring during the disease process. Gene-expression profiling confers a "snapshot" of cellular activity providing information on mechanisms mediating disease changes, elucidating possible pathways involved and can also generate diagnostic gene sets. In AS and spondyloarthritis (SpA) a number of recent studies have defined transcriptional profiles generated from peripheral blood mononuclear cells (PBMCs) isolation requiring immediate sample processing, which is not suitable for larger multicentre studies and limits the viability of such an approach [7]. An alternate approach is to use whole blood samples collected using PAXgene technology which preserves the integrity of the RNA even with limited storage at room temperature allowing delays in transport and handling to occur with minimal RNA degradation [7].

In the current study, we undertook a whole-genome microarray approach to identify a genomic profiling in a Portuguese case-control collection, using RNA from peripheral blood collected using the PAXgene collection system, and validated these gene-expression changes in an independent larger sample cohort using quantitative PCR (qPCR). Our goal was to test whether genomic profiling in such cases, using the more practical PAXgene Blood RNA System®, could distinguish AS cases from healthy controls, and identify genomic pathways likely to be involved in AS pathogenesis.

Materials and methods

Study subjects

The microarray-based discovery study was performed using samples from 18 AS patients, diagnosed according to the modified New York criteria [8], and 18 gender- and age-matched healthy controls (±5 years). Included patients had Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) scores >4 and Bath Ankylosing Spondylitis Functional Index (BASFI) scores >4. All patients were receiving only NSAIDs and/or sulphasalazine. No TNF, corticoid or methotrexate treated patients were included. Details of the study subjects are shown in Supplementary Table S1 in Additional file 1.

Candidate genes were validated in a second larger cohort of another 78 AS patients and 78 age and sex matched controls (full details in Supplementary Table S2 in Additional file 2).

Peripheral blood samples were collected into PAXgene Blood RNA System® tubes (Qiagen, Doncaster, VIC, Australia) and stored according to the manufacturer's recommendations [9]. This study was approved by the Ethics Committees of the participating centres, and written informed consent was obtained from the individuals involved in this study.

RNA processing and array analysis

Total RNA was extracted from whole blood samples according to the standard PAXgene protocol, quantified and the integrity assessed by Agilent 2100 BioAnalyser (Agilent, Santa Clara, CA, USA). Only samples with a RNA integrity number above 7.5 were used. To minimize the effects of Globin RNA transcript over-representation, samples were processed with Ambion GLOBINclear® (Applied Biosystems, Mulgrave, VIC, Australia) according to the manufacturer's protocol. cRNA was generated from 500 ng of total RNA using the Illumina TotalPrep cRNA Amplification Kit® (ABI) and hybridized to Human HT-12 V3 Expression BeadChips (Illumina, San Diego, CA, USA). Array data were processed using the Illumina GenomeStudio software, transformed by variance stabilization transformation (VST) [10] and normalized by robust spline normalization [11] using Lumi [12]. Quality control using principal components analysis showed four samples to be outliers and further investigation revealed technical issues with the processing of these samples and thus they were excluded from the analysis.

Gene expression analysis was performed in BRB-ArrayTools [13]. Differentially expressed genes were identified by unpaired t-test with multivariate permutation correction. Gene ontology analysis was carried out in BRB ArrayTools using a LS permutation test which finds gene sets that have more genes differentially expressed among the classes than it would be expected by chance using 100,000 random geneset permutations to compare to the chosen geneset.

Candidate gene validation using quantitative reverse transcription polymerase chain reaction

Candidate genes were identified from the array studies based upon their fold-change between AS and control, the *P*-value of this difference and their potential biological relevance to AS. Candidate genes were assayed using real-time quantitative PCR-based (qPCR) predesigned TaqMan Low Density Array Cards (TLDA). The TLDA cards had 48 predesigned Taqman qPCR assays, which utilise MGB probes with FAM dye, arrayed in a 384-well format allowing four samples to be assayed per TLDA. Forty-seven candidate genes selected from the whole genome arrays together with a housekeeping gene (*18s*) were arrayed. cDNA was generated from 1 μg of total RNA using the Bioline cDNA synthesis Kit (Bioline, London, UK) according to manufacturer's instructions. qPCR was carried out using SensiMix dT

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RT-PCR reagent (Quantace, Sydney, QLD, Australia) under the following conditions; 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 s and 60°C for 60 s [14].

Data were normalized using the housekeeping gene, *18S*, included on the card and quantified using the $2^{-\Delta CT}$ [15]. Data were analysed with the Mann-Whitney test and *P*-values <0.05 were considered significant (SPSS v17.0, Chicago, IL, USA).

Results

Differential gene expression in AS patients and controls

From a total of more than 48,000 probes on the array, 18,159 were found to be expressed in at least one sample and were included for analysis. To estimate the degree of gene expression variation driven by disease status, we undertook unsupervised hierarchical clustering using the top 3% most variably expressed genes without reference to disease status. Clustering with this non-biased geneset gave good delineation between the controls and AS patients with only six controls and five AS patients misclassifying (Figure 1). To identify the genes specifically differentially expressed between patients and controls, we carried out an unpaired T-test corrected for multiple comparisons. A total of 648 probes were considered significantly differentially expressed (80% confidence level of false discovery rate, with 10% false positives) with 204 of these probes (corresponding to 190 genes) having *P*-values <0.0005 (Supplementary Table S3 in Additional file 3). The magnitude of differential expression observed was generally low, with only three genes showing a significant fold-change >2 (with the maximum fold-change being 2.21) and 29 genes >1.5 fold-change. Of the 204 probes, 89 probes were upregulated and 115 downregulated. Our microarray data are available in a public repository, Gene Expression Omnibus (GEO), with the accession number [GEO:GSE25101].

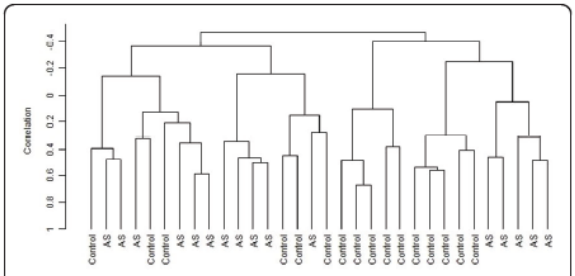

Figure 1 Clustering on top 3% most variable genes. Unsupervised hierarchical clustering based upon the top 3% most variable genes (585 genes) showed good delineation between the patient and control samples with six controls and five AS samples misclassifying. The non-perfect classification suggests non-disease related factors also drive the gene expression patterns.

We then selected 47 of the differentially expressed genes for validation by qPCR (Table 1) based upon their *P*-value, fold-change and biological relevance.

Quantitative RTPCR validation

Expression levels of the 47 selected genes were confirmed by qPCR in a second sample set consisting of 78 patients and 78 age and gender matched controls. A total of 28 of the 47 genes showed a similar trend in differential expression between AS and control samples as the array data. Of these, 14 of the 47 considered genes were validated with significant *P*-values with 13 downregulated 1.4 to 2.2-fold and only 1 upregulated (1.6-fold) (Table 2).

Gene ontology analysis

Gene ontology (GO) analysis on the dataset showed two key immune-associated pathways to be altered, "negative regulation of adaptive immune response" and several ontologies affecting "thymic T cell selection" both with *P*-values <0.005 (Table 3).

Discussion

Gene expression profiling in disease reveals the underlying gene activity changes contributing to the disease process. This information provides insight into the tissue changes during the disease development and enables targets for therapeutic intervention to be identified. Secondly, strong, consistent gene expression changes can be utilized to generate diagnostic algorithms to identify early-stage disease before significant tissue damage has occurred.

However, in SpA, and in AS in particular, only a small number of case-control genomic profiling studies have been undertaken. The early studies involved low sample numbers and poor genome coverage and were very heterogeneous in terms of methodology, including the source of mRNA studied [16-19]. More recently four studies using genome-wide microarrays showed interesting results regarding SpA physiopathology and biomarker identification [20-23]. Of these, two were PBMC based [20,21] and in two the RNA was isolated from unfractionated peripheral blood [22,23]. However, all these studies did confirm that AS/SpA cases could be reliably distinguished from healthy controls using genomic profiling [20-23].

Although PBMCs have been widely used in autoimmune disease gene expression profiling studies, PBMCs do not represent an ideal tissue source for larger scale multicentre studies requiring extensive downstream processing soon after collection. PAXgene tubes enable whole blood to be collected directly into an RNA-preservative which stabilises the RNA from degradation for up to three days at room temperature, long enough for

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Table 1 Selected genes for validation by qPCR

Gene symbol	Fold-change (AS/Cont)	Parametric *P*-value
CX3CR1	0.58	3.45E-04
DGKQ	0.62	1.45E-05
SPOCK2	0.65	3.07E-04
SBK1	0.66	1.69E-04
GZMM	0.67	3.39E-04
CDC25B	0.67	2.00E-04
CLSTN1	0.68	4.53E-04
ITGB7	0.68	5.63E-04
PTPN1	0.69	1.50E-06
EP300	0.70	1.26E-04
DOCK10	0.70	1.73E-04
MAPK8IP3	0.70	3.37E-04
BCL11B	0.70	5.88E-04
DNMT1	0.71	6.46E-05
XPC	0.71	5.10E-06
PPP2R1A	0.71	1.73E-04
IL27RA	0.72	4.40E-06
MCM3	0.73	4.24E-04
SYF2	1.34	5.94E-04
PPP2R3C	1.36	1.07E-03
NGFRAP1	1.37	1.87E-04
ZMAT2	1.38	1.58E-04
MYL6	1.41	7.94E-05
S100A8	1.43	6.09E-04
GMFG	1.43	3.91E-05
VAMP5	1.44	4.21E-04
CKLF	1.46	4.30E-04
SHFM1	1.47	8.62E-04
ATG3	1.49	6.10E-04
MRPS18C	1.49	5.71E-04
CLEC4D	1.52	3.47E-04
UBL5	1.52	8.93E-05
AIF1	1.52	5.19E-04
PDCD10	1.54	2.45E-03
NDUFS4	1.54	5.93E-04
SF3B14	1.54	2.39E-04
HMGB2	1.57	3.41E-04
UQCRB	1.63	2.33E-04
TXN	1.68	4.39E-05
CMTM2	1.74	4.78E-04
CIP29	1.75	1.38E-04
CHMP5	1.78	6.80E-04
PSMA4	1.80	8.45E-05
NDUFB3	1.88	1.25E-04
LSM3	1.99	6.72E-04
GNG11	2.15	1.41E-04
CCDC72	2.21	9.64E-05

Genes ($n = 47$) for validation by qPCR were selected from the 648 probes significantly differentially expressed in microarrays based upon their *P*-value, fold-change and biological relevance. AS, Ankylosing spondylitis; qPCR, quantitative polymerase chain reaction.

Table 2 Validated genes by qRTPCR

Parametric *P*-value	Fold-change	Gene symbol
2.90E-06	0.4457398	BCL11B
1.30E-06	0.4597414	DNMT1
2.50E-06	0.5009603	CDC25B
1.94E-05	0.5214546	CLSTN1
0.008539	0.5323253	VAMP5
1.81E-05	0.5435122	DOCK10
0.0005221	0.5693345	SPOCK2
0.0012085	0.5831974	ITGB7
0.001398	0.6124764	MCM3
0.0206755	0.6632639	CX3CR1
0.0026443	0.6749132	PTPN1
0.0034889	0.6847808	EP300
0.0125026	0.7141221	PPP2R1A
0.0184087	1.6090812	CLEC4D

Of the 47 considered genes, 14 were validated with significant *P*-values ($P < 0.05$). q-RTPCR, quantitative reverse transcription polymerase chain reaction.

the samples to reach a safe frozen depository [9]. However, differences in the transcriptional profile between PBMC and PAXgene derived RNA have been demonstrated. These differences are due to the different cell populations targeted as well as the extra processing steps required for PAXgene samples to prevent globin mRNA overrepresentation affecting microarray sensitivity [9,24]. The study reported here has a number of strengths in that it represents a multicenter study involving ethnically homogeneous patients with AS (defined according to the modified New York criteria) and excluded patients on anti-TNF agents. Use of a primary dataset for candidate gene identification (by microarrays) followed by validation by qPCR in an independent dataset provides additional robustness.

Using the most variably expressed genes for unsupervised clustering gave us an estimate of the proportion of the gene expression variation driven by disease status. That only six controls and five AS samples were misclassified indicates the major driver of gene expression as disease status. However, the fact that not all samples classified correctly also indicates other factors driving gene expression variation. Such factors may be differences in blood collection, storage and transport protocols that can arise in multicentre studies. However, none of these effects were robust enough to cluster independently when tested (data not shown).

We validated 14 differentially expressed genes between AS patients and healthy controls. A number of these genes have well-documented inflammatory roles or an action on bone/cartilage metabolism. Moreover, GO analysis showed two key immune-associated pathways to be altered, "negative regulation of adaptive immune

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Table 3 Gene ontology analysis

GO category	GO ontology	GO term	LS permutation *P*-value
GO:0002820	BP	Negative regulation of adaptive immune response	0.00001
GO:0033077	BP	T cell differentiation in the thymus	0.00001
GO:0043383	BP	Negative T cell selection	0.00001
GO:0045061	BP	Thymic T cell selection	0.00001

The dataset showed two key immune-associated pathways to be altered, "negative regulation of adaptive immune response" and several ontologies affecting "thymic T cell selection" both with *P*-values <0.005.

response" and several ontologies affecting "thymic T cell selection". Interestingly the GO analysis indicated a "negative" regulation of the immune system. This agrees with a previous expression profiling recently reported in PBMCs from AS patients [20] and is also consistent with the "reverse IFN gamma signature" reported by another group studying macrophages from AS patients [19].

A possible reduced immune response also correlates with the downregulation of *PTPN1* and *DOCK10*, which are both involved in mediating IL4 actions [25]. Protein tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed enzyme shown to negatively regulate multiple tyrosine phosphorylation-dependent signalling pathways, including IL4 signalling [26]. Dock10 is also regulated by IL4 in B cells [27]. This is of particular interest as IL4 may play a role in AS pathogenesis. Interleukin 4 (IL4), a 20-kDa product from activated T lymphocytes, has a variety of stimulatory and inhibitory actions on B and T cells [25,28-30]. Recent studies have also indicated a potential role for IL4 producing CD8+ T cells in the pathogenesis of AS. Although CD8+ T cells are predominately associated with the production of 'Th1' cytokines, such as IFNgamma, there is now good evidence that some subsets of these cells can also produce 'Th2' cytokines such as IL4, IL5 and IL10 [31]. The potential functions associated with IL4-producing CD8+ T cells are as yet unclear but the subtype CD8+/TCR alpha beta + T cells, with a regulatory phenotype and function (expressing CD25+, CTLA4+, Foxp3+, but negative for IFNgamma and perforin), were previously described in peripheral blood of AS patients [32]. These results were confirmed in a recent study suggesting an altered pattern of CD8+ T cell differentiation in AS and in *HLA-B27*+ healthy individuals [33]. This predisposition to generate IL4+CD8+ T cells may play a role in pathogenesis of SpA [32,33].

In addition, the activation of the innate immune system has been proposed to play an important role in AS inflammation. Dysregulation of Toll-like receptor (TLR)-related pathways (an upregulation of TLR4 and TLR5), involved in innate immune response, have been described [23]. Interestingly we also identified increased expression of TLRs 4 and 5, together with TLR1 but the significance was marginal and, therefore, not followed up. However, an upregulation of C-type lectin domain family 4, member D (CLEC4D), another gene involved in the innate immune response, was seen. CLEC4D has been found to be expressed in a monocyte/macrophage restricted manner, and although no ligand or biological function has as yet been described, the receptor has been shown to be upregulated at the transcript level in a number of disease settings, similarly to two others members of the family, Mincle and Dectin-2. They are able to recognize and promote pathogen clearance and induce inflammatory signals [34]. This process seems to follow the Syk and caspase recruitment domain protein (CARD9) pathway which was recently implicated in a mouse model of SpA [35].

Changes in *SPOCK2* (osteonectin) and *EP300* provide interesting insights into AS progression. *SPOCK2*, also known as Sparc/osteonectin, was implicated, in a recent study, as a discriminator between SpA and healthy controls [22] and has been hypothesised to play roles in the regulation and production, assembly, or maintenance of matrix turnover in cartilage [36,37]. In this process TGFbeta and IFNgamma exert antagonistic effects, and play important roles in the physiologic regulation of extracellular matrix turnover. TGFbeta positively regulates *COL1A2* through the cellular Smad signal transduction pathway, contrary to IFNgamma which downregulates *COL1A2* through Stat1. Interestingly, the protein produced by EP300 belongs to the group of nuclear p300/CBP transcriptional coactivators for both Smad3 and Stat1a, and integrates signals that positively or negatively regulate *COL1A2* transcription [38]. In addition, the downregulation of *EP300* may promote a pro-inflammatory status [39] contributing to cartilage degradation. The protein phosphatase 2, regulatory subunit A (*PPP2R1A*) has also been shown to play a role in TGFβ-mediated regulation of Smad3-activated genes [40]. Finally, transactivated p300, controlled by phosphoinositide-3 kinase (PI3K)/AKT, is an important transcriptional co-activator of Sox9 [41], which modulates the expression of the major extracellular matrix component, aggrecan. Not surprisingly, altered *EP300* expression has been associated with the Wnt pathway, a key mediator of bone formation as well as cartilage

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alterations in osteoarthritis [42]. Downregulation of these genes might lead to a loss of matrix integrity thereby accelerating tissue damage. PTP1B has also been shown to induce apoptosis in chondrocytes, thus downregulation might result in increased chondrocyte numbers contributing to joint damage as has been seen in osteoarthritis [43]. Both EP300 and DNA (cytosine-5-)-methyltransferase 1(*DNMT1*), mediate STAT3 functionality [44] which has also been associated in genetic studies with AS [45]. Decreases in STAT3 signalling might also contribute to the hypothesised reduction in immune response [46].

CX3CR1, which plays a pro-inflammatory role in RA, was also downregulated. In RA, CX3CR1, and its ligand CX3CL1, drives chemotaxis of pro-inflammatory monocytes to inflammatory sites [47]. Decreased expression of this gene in AS may reflect the fundamental differences in disease processes between AS and RA.

Beta 7 integrins have been shown to play a role in chronic ileitis [48,49] a common clinical feature of SPA. In our study, beta 7 integrin (*ITGB7*) is under-expressed again possibly reflecting a decreased immune response in AS.

The specific function of several other genes, as *BCL11B*, *CDC25B*, *VAMP5*, *MCM3*, *CLSTN1* have not yet been determined and their potential involvement in disease processes needs additional research.

Conclusions

We have validated a gene expression signature for AS from whole blood and identified strong candidate genes that may play roles in both the inflammatory environment and bone and cartilage effects. Future studies are needed to confirm some of the possible interactions suggested by this study.

Additional material

Additional file 1: Supplementary Table S1: Characteristics of subjects involved in microarrays study. BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Spondylitis Metrology Index; mSASSS, modified Stoke Ankylosing Spondylitis Spinal Score.

Additional file 2: Supplementary Table S2: Characteristics of subjects involved in TLDA study. AS (n = 78) and healthy controls (n = 78). No significant differences for age and sex between groups.

Additional file 3: Supplementary Table S3: Genes differentially expressed between AS patients and controls by microarrays. A total of 648 probes were considered significantly differentially expressed (80% confidence level of false discovery rate with 10% false positives).

Abbreviations

AS: ankylosing spondylitis; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; BASMI: Bath Ankylosing Spondylitis Metrology Index; GO: gene ontology; mSASSS: modified Stoke Ankylosing Spondylitis Spinal Score; PBMCs: peripheral blood mononuclear cells; qPCR: quantitative polymerase chain reaction; q-RTPCR: quantitative reverse transcription polymerase chain reaction; SpA: spondyloarthritis; TLDA: TaqMan Low Density Array Cards.

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Authors' contributions

FMPS and GT participated in the conception and design of the study, carried out the lab work, performed the data analysis and drafted the manuscript. DL, HGP, JCB and MAB participated in the conception and design of the study and critically revised the manuscript. JEF critically revised the manuscript. FMPS, MM, AFM, JC, HS, AB, FG, PP and MC were involved in primary data collection. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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3. DISCUSSION AND CONCLUSIONS

3.1. LESSONS FROM EPIDEMIOLOGICAL ANALYSIS

3.1.1. Metrological and epidemiological data relevance

The Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) [Garret *et al.*, 1994], the Bath Ankylosing Spondylitis Global Score (BASG) [Calin *et al.*, 1994], the Bath Ankylosing Spondylitis Functional Index (BASFI) [Jones *et al.*, 1996], as well as the Bath Ankylosing Spondylitis Metrologic Index (BASMI) [Jenkinson *et al.*, 1994] were developed in the 90's at the Royal National Hospital for Rheumatic Diseases in Bath, UK. These indices are widely used by the scientific community and are currently key-elements in assessing and monitoring activity, functioning, and mobility repercussions of AS. In addition, several components of the BASDAI, BASFI, BASG and BASMI are included in the Assessment of SpondyloArthritis Society (ASAS) score set for symptom modifying antirheumatic drugs and physical therapy [van der Heijde *et al.*, 1999], the ASAS 20, 40, 60, 5/6 improvement criteria and partial remission criteria [Anderson *et al.*, 2001]. Several components of the BASDAI are also parameters used in the ASDAS [Lukas *et al.*, 2009; van der Heijde *et al.*, 2009; Machado *et al.*, 2011].

Worldwide distribution of AS and the importance of these indices set the need for a homogenous assessment. Therefore, these indices have been cross-culturally adapted for use in numerous languages. Despite their importance, these indices haven't validated in Portuguese language yet. For this reason, the original English versions were translated, culturally adapted, and validated. The Portuguese adapted versions of these indices

maintained all the properties of the original English versions of the instruments. Disease activity, functioning, and severity in Portuguese-speaking patients with AS may be now appropriately evaluated with these versions of the original instruments (Anexe II) [Pimentel-Santos *et al.,* 2012b]. Not only Portuguese patients can be more correctly and accurately assessed, but also more precise comparisons between patients of different ethnicities can be carried out. The above-mentioned instruments can, therefore, now be applied in clinical practice and for research purposes with great benefit for both Portuguese and international scientific communities.

The reference centile charts developed for Portuguese population for BASDAI, BASFI, BASMI and mSASSS represent, in this context, a useful contribution for ethnic comparisons [Pimentel-Santos *et al.,* 2012c]. Unfortunately, at this point only the English reference centile [Taylor *et al.,* 1998] charts are available to carry out such comparisons. However, the charts do provide some descriptive information regarding disease activity, functional impairment, metrology and radiological impact in AS, in the same population, over time. Furthermore, these reference charts, with visual representation may improve patients' understanding of the disease, which may improve their compliance to treatment. After proper validation (ongoing project), they may be applied in clinical practice on an individual basis in order to follow any given patient over time. The possibility of adding this method to others, such as ASAS or ASDAS, in disease monitoring may be useful for patients as well as for clinicians. Presently, reference centile charts performed in Portuguese population seem to confirm the interpretation that arose from the English population - women appear to be more functionally impaired than men and have higher disease activity, regardless of better metrology (and, in our cohort, less radiological change) [Taylor *et al.,* 1998; Pimentel-Santos *et al.,* 2012c]. Overall, these results

show differences between men and women in the phenotypic expression of AS. This topic will be discussed in more detail below.

3.1.2. Epidemiological comparison between populations and the influence of gender

There are limited epidemiological data on AS in Portugal despite its prevalence. It is important to have a picture of the disease to identify major concerns and to create appropriate intervention strategies. Moreover it allows the comparison of data from our population with other ethnic groups and a better characterization of the disease when different subgroups are considered.

In the present work, a comparison of clinical features, activity, functional, metrological, and radiographic scores was also carried out between males and females. In fact, the aim of this research was not only to characterize AS in Portugal, but also to make comparisons with other ethnic groups (Table 2) and to understand the potential influence of gender on phenotypic characteristics of the disease and its prognosis (Table 3).

AS usually begins in late adolescence or early adulthood; onset after 40 years is very rare [Zink *et al.*, 2000; Reveille, 2008]. In agreement with this, the mean age of disease onset in Portugal is 26.5 years, with juvenile onset (under 16 years) in 10.6% of patients and late onset (older than 40 years) in 10% of them. Regarding the gender, mean age of disease onset in males is slightly lower (25.8 years) than in females (27.5 years), a difference that is not nevertheless statistically significant. These results are similar to those reported in other countries [Geirsson *et al.*, 2010]; although there are some variability that in some cases reaches statistically significant difference [Aggarwal & Malaviya, 2009; Kim & Kim, 2010]. However, there are descriptions that the average age at disease onset is slightly earlier in females than in males [Feldtkeller *et al.*, 2003; Lee *et al.*, 2007].

Table 2: Clinical comparison of different ethnic groups with AS published after 2000.

	Portugal	Spain	Brazil	Argentina	India	Korea	Greece
Author	Pimentel-Santos *et al.*, 2012c	Garcia *et al.*, 2008	Sampaio- Barros, 2011	Buschiazzo *et al.*, 2011	Aggarval & Malaviya, 2009	Kim & Kim, 2010	Alamanos *et al.*, 2004
n	369	1422	736	86	70	830	113
Male:Female, n(%)	232/137(62.8/37.1)	1069/353(75.2/24.8)	570/166(77/23)	76/10(88.4/11.6)	59/11(84.3/16.7)	732/98(88.2/11.8)	93/20
Age, mean(SD)	45.4(13.2)	48.4(12.7)	-	40	23.6(8.8)	33.2(10.1)	-
Age symptom onset, mean(SD)	26.5(10.8)	26.9(10.4)	27.7(11.4)	-	23.6(8.8)	20.9(8.1)	30.5(10.7)
Diagnosis delay, mean(SD)	7.6(9)	13.5(10.5)	-	3*	6.9(5.2)	-	-
Family history	17.6%	20.2%	16.9%	20.9%	52.9%	19.6%	-
Extra-articular manifestations	35.2	-	-	-	29.6	-	13.3
AAU	33.6%	-	-	-	25.7%	29.7%	-
BASDAI, mean(SD)	4.2(2.3)	4.1(2.3)	4.6(3.3)	3.9*	3.2(1.8)	-	-
BASFI, mean(SD)	4.1(2.7)	3.9(2.7)	4.9(2.7)	4*	2.3(2.0)	-	-
BASMI, mean(SD)	4.0(2.5)	-	-	-	3.15(2.3)	-	-
HLA-B27	80.5%	-	-	-	92.9%	94.8%	80.5%

*median; AAU acute anterior uveitis

Table 3: Clinical comparison between females and males in different ethnic groups published after 2000.

	Portugal		**USA**		**India**		**Korea**		**Brazil**		**Iceland**	
Author	Pimentel-Santos *et al.*, 2012c		Lee *et al.*, 2007		Aggarval & Malaviya, 2009		Kim & Kim, 2010		Sampaio- Barros, 2001		Geirsson *et al.*, 2010	
Gender	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
n	232	137	302	100	59	11	732	98	124	23	145	78
Age, mean(SD)	45.7 (13.5)	44.9(13.9)	55.5(10.6)	53.0(11)	-	-	32.9(9.9)	35.4(9.9)	-	-	-	-
Age symptom onset, mean(SD)	25.8(10.8)	27.5(10.8)	23.6 (7.9)*	21.5(7.3)*	22.3(8.5)*	30.0 (8)*	20.6(8)*	23.3(8.9)*	-	-	23.6(8.4)	24.1(8.9)
Diagnosis delay, mean(SD)	7.1(9)	8.3(9)	-	-	6.5(4.7)	8.6(6.6)	-	-	-	-	8.3(7.7)	9.6(10)
Family history	15.2%	21.9%	24.6%	41%	-	-	18.3%	29.3%	15.3%	8.7%	-	-
AAU	34.7%	33.9%	42%	44%	16.9%	72.7%	28.2%	40.8%	15.3%	8.7%	37.9%	30.8%
BASDAI, mean(SD)	3.7(2.2)	4.9(2.3)	-	-	-	-	-	-	-	-	-	-
BASFI, mean(SD)	3.8(2.6)	4.5(2.7)	4.1	4.2	-	-	1.8(1.9)	1.8(2)-	-	-	-	-
HLA-B27	83.2%	76.4%	88.7%	84%	-	-	94.9%	93.7%	78.2%	78.2%	85.5%	80.8%

*median; AAU acute anterior uveitis

Until now, no sound explanations were proposed for these divergent results in AS. One may speculate that different methodologies between studies may account for such differences in results; likewise, the importance given to symptoms as a function of gender may also not be negligible. The age at diagnosis in Portugal (34.1 years) seems to be significantly lower in males than in females (33 years *vs* 35.8 years, $p<0.05$, respectively), which is due both to lower ages of symptom onset, as previously discussed, and a lower diagnosis delay (7.1 years *vs*. 8.3 years, $p>0.05$, respectively) in males. The delay identified in the overall Portuguese cohort (7.6 years) and between genders is similar to that reported recently in other countries [Reed *et al*., 2008; Aggarwal & Malaviya, 2009; Geirsson *et al.*, 2010]. Based on these and other earlier studies [Calin *et al*., 1988], female patients have shown to have a longer delay (1-2 years) before their diagnosis is established although in the majority of these studies no statistical differences between genders were reported; likewise, Portuguese population yielded similar findings. This tendency might be related with misconception among health care providers that AS is a disease of men leading to a widespread insensitivity to recognize AS in women.

Interestingly enough, the present work showed a lower male:female ratio of 1.7:1. Similars values (1.9:1) were reported in Iceland [Geirsson *et al.*, 2010] and in Switezerland [Brunner *et al.*, 2002]. Slightly high values of 3-4:1 was reported in USA [Lee *et al.*, 2007], Brazil [Sampaio-Barros *et al.*, 2011], Spain [Garcia *et al.*, 2008] which is quite different from reports of 8-10:1 in some Asian populations [Kim & Kim, 2010; Zeng, 2003] or in earlier studies [Sigler *et al.*, 1971; Wright & Moll, 1973]. One may hypothesize that different methodologies (inclusion/exclusion criteria, community *vs* hospital based studies) may

explain these differences; regional variability in terms of genetic background and in terms of environment may also have some influence.

A general tendency for decrease of male:female ratios over time, may be related to better knowledge that both patients and scientific community have about AS and also to a better access to diagnostic tools. On the other hand, possible gender differences in disease patterns may confer additional diagnosis difficulties. The perception that diagnosis of AS is not as easily reached in women as it is in men - and that disease may therefore be underdiagnosed in women - was the reason to carry out an extensive evaluation of symptoms, disease activity, functioning, and metrologic repercussion of AS patients in this study.

The Portuguese data seems to report a traditional AS patient group having lower back pain (42.3%) as an onset symptom. At the time of assessment 49.9% had axial disease, 2.4% peripheral disease, 40.9% mixed disease and 7.1% isolated enthesopatic disease. Peripheral joint involvement is usually described as an onset symptom in 10–20% (11.1% in Portugal, non-published data) of patients, and 30-40% of all patients have this symptom at some point of their disease course [Ginsburg & Cohen, 1983; Gran, 1985a; Eastmond, 1996]. Hips and shoulders are the most frequently implicated extra-spinal joints in AS; they are concerned at onset in up to 15% of patients and at some stage of disease in up to 35% [van der Linden & van der Heijde, 2000]. According to a systematic review of earlier studies women had less severe involvement of the spine and more peripheral arthritis [Levitin & Davis, 1975; Resnick *et al.*, 1976; Braunstein *et al.*, 1982; Marks *et al.*, 1983; Will *et al.*, 1990; Eustace *et al.*, 1993; Jimenez-Balderas & Mintz, 1993] than males. When rachis is affected in women the involvement tends to be more pronounced in cervical segment [Tyson *et al.*, 1953; Resnick *et al.*, 1976; Marks *et al.*, 1983; Maldonado-Coco *et al.*, 1985]. However, a number of

methodological issues limits the conclusions that can drawn from these studies. Interestingly in Portugal, the proportion of females (53.3%) with peripheral involvement seems to be higher than in males (41.7%) in accordance with other recent studies [Lee *et al.*, 2007; Geirsson *et al.*, 2010]. All together these results reinforce earlier studies conclusions despite conflicting results from India [Aggarwal & Malaviya, 2009] and Korea [Jung *et al.*, 2010], where no differences in peripheral joint involvement between genders were found. Other differences between male and female might be evident in extra-articular manifestations which were experienced by 35.2% of the Portuguese patients, being AAU (33.6%), the most common feature [Pimentel-Santos *et al.*, 2012c]. This is in agreement with the literature reporting AAU as the most common extra-articular manifestation in AS occurring in up to 40% patients [Reveille, 2008]. Most studies [Gran *et al.*, 1985b; Rosenbaum, 1989; Rothova *et al.*, 1992; Jimenez-Balderas & Mintz, 1993; Tay-Kearney *et al.*, 1996; Lee *et al.*, 2007; Geirsson *et al.*, 2010] didn't show any significant differences in AAU between genders, as it was the case of Portugal (35% male *vs.* 33.3% female, $p>0.05$). Some conflicting results are reported in studies performed in Asian population showing that AAU in females was significantly more common [Aggarwal & Malaviya, 2009; Kim & Kim, 2010]. It's possible that ethnicity may play a role in such differences but methodological differences might not be excluded.

Overall, there aren't in the literature convincing explanations regarding different phenotypic characteristics between genders. If current data on sex steroid hormones provide no straightforward explanation for the male predominance in AS [Straub *et al.*, 2002] no information is available regarding its influence in clinical manifestations. Their role is still not completely understood [Masi, 1992; Giltay *et al.*, 1999; Straub *et al.*, 2002]. Alternative

explanations for these differences may be found in sexual chromosomes. However, no linkage was found between the X chromosome and susceptibility to AS [Hoyle *et al.*, 2000; Zhang *et al.*, 2004]. No evidence comes from recent GWAS performed where no association has been established between sexual chromosomes and AS susceptibility [Burton *et al.*, 2007; Evans *et al.*, 2011]. No data are available regarding disease phenotype. Further studies are required to clarify hormones and sexual chromosomes in disease pathogenesis.

In this context AS / SpA family history or *HLA-B27* positivity becomes particulary interesting as it might help in the analogy process, thus contributing to a decrease in the diagnosis delay. In this topic there are once again, discrepant results in published studies. It seems to be a tendency for women to report family history of AS in first-degree relatives more often than males. In some studies the difference reaches statistical difference [Lee *et al.*, 2007; Kim & Kim, 2010], but not in others [Aggarwal & Malaviya, 2009]. In Portugal, family history was reported in 17.6% of patients in the overall cohort; although females reports family history more often than males (15.2% males *vs.* 21.9% females, $p>0.05$), the difference is not statistically significant. Thus the finding that more females have positive family histories could suggest that the diagnosis of AS may be made more often in females when suspiction for AS is heightened. This may partially explain lower male:female ratios in such populations, but it doesn't seems to be a reasonable explanation for Koreans [Kim & Kim, 2010]. In addition similar proportions of *HLA-B27* positivity were described in both genders [Hill *et al.*, 1976]. *HLA-B27* is positive in 80.3% of AS Portuguese patients, a value similar to the one found in Greece (80.5%) [Alamanos *et al.*, 2004] but lower than the one referring to India (92.9%) [Aggarwal & Malaviya, 2009] or to Korea (94.8%) [Kim & Kim, 2010]. In Portugal, proportion in males and females was 82.4% *vs.* 75.4% ($p>0.05$), respectively. Similar results

were described in USA (88.7% *vs.* 84.0%, p>0.05) [Lee *et al.*, 2007], in Korea (94.9% *vs.* 93.7%, p>0.05) [Kim & Kim, 2010], in Iceland (85.5 *vs.* 80.8 %, p>0.05) [Geirsson *et al.*, 2010] and in Brazil (78.2% *vs*. 78.2%, p>0.05) [Sampaio-Barros *et al.*, 2001]. Although it is not possible from these studies to estimate the real impact of HLA-B27 positivity on diagnosis delay, a study [Feldtkeller *et al.*, 2003] showed that the delay is significantly longer in HLA-B27 negative than in HLA-B27 positive patients.

In terms of activity and severity of disease, data gathered in this study showed high mean values of BASDAI (4.2), BASFI (4.1), and BASMI (4.0). Although these values are similar to those found in other populations, it also means Portuguese patients have, on average, high levels of activity and disease severity. In other words, this means that the disease is not adequately controlled in a large number of patients. Considering gender, as previously discussed, it has been increasingly recognized that AS is not so uncommon in females. Furthermore, the symptoms they experience are at least of similar severity [Zink *et al.*, 2000], or even worse, than in males [Pimentel-Santos *et al.*, 2012c]. In fact, as previously reported in English and presently in Portuguese patients, females have more self-reported disease activity and more functional impairment than males, despite their better metrology [Taylor *et al.*, 1998] and a better radiological evaluation (mSASSS) [Pimentel-Santos *et al.*, 2012c]. These differences were valid for results as a whole, but also when different periods of disease duration were considered [Pimentel-Santos *et al.*, 2012c]. Less structural changes in women than in men has been highlighted previously [Feldtkeller & Braun, 2000; Lee *et al.*, 2007] but for a given degree of radiographic damage, women reported more functional limitations [Lee *et al.*, 2007]. However, the relationship between accumulation of spinal radiographic damage and corresponding function in AS remains unknown.

Overall, these data highlight the idea that there are different phenotypic patterns in AS according to the gender. In addition the relatively slow clinical and radiological development in women [Jimenez-Balderas & Mintz, 1993], potentially contribute to a more difficult recognition, which in turn leads to misdiagnosis in early stages of the disease. It is important to considerer the overall diagnosis delay of eight years, which appears to be very long. Several potential explanations have been identified:

- One of them is lack of sensitivity of the modified New York Criteria when used in clinical practice. Therefore it would be interesting to evaluate in a few years the changes introduced by new ASAS criteria for axial and peripheral spondylarthritis [Rudwaleit *et al.*, 2009b, 2009c; Rudwaleit, 2010a, 2010b];

- Another reason may be the non-recognition of inflammatory back pain by patients and/or caring physicians and/or difficulty in accessing rheumatology care. In order to solve the problem of lack of information, the Portuguese Society of Rheumatology has recently promoted several campaigns in different media. In order to improve the coverage of Rheumatology care, the General Directorate of Health (DGS) has likewise implemented a network of rheumatology referral hospitals according to a National Program Against Rheumatism ("**Programa Nacional Contra as Doenças Reumáticas**"). As a consequence, several rheumatology units have been established in different regions of Portugal. It will be interesting to evaluate in the next few years the impact of all these health care strategies in terms of disease activity, severity and HRQoL parameters.

Discussion of results has been articulated around diagnosis delay in order to bring out strategies for an earlier diagnosis and, therefore, avoid delayed therapy onset, which in turn might be an additional detrimental factor for prognosis [O'Dell, 2002]. A growing body of

evidence emphasizes that prognosis improves when patients with early RA or undifferentiated arthritis are referred to, and managed by, experts in aggressive rheumatologic care [Cush, 2007]. Additionaly, there is a period in early RA onset ("window of opportunity" concept [O'Dell, 2002]) during which there is great potential for remission and/or for obtaining a better clinical response. Although the scientific community extrapolated these ideas to SpA; clinical evidence still lacks in this area. Interestingly, in the analysis performed in Portuguese population, no statistical difference was found between patients with early and late diagnosis in terms of disease activity, function, metrology and radiological repercussion, which suggests that early diagnosis (and subsequent early therapy onset) may not indicate better prognosis. However, these results must be interpreted in the light of the cross-sectional, observational study design. As commented in the paper, one possible explanation may be that early diagnosis is associated with a more active disease, which decreases the potential benefit of an early intervention rather than simply reflecting paucity of benefit of an early treatment. On the other hand, many patients in this cohort were diagnosed as AS cases more than 10 years ago, when therapeutic strategies were clearly different from those currently available. The relevance of the delay in diagnosis should prompt a detailed evaluation in future studies.

In this context it would be interesting analyse therapeutic approach of the Rheumatology community. From the BASDAI charts analysis it is interesting to confirm previous findings that AS remains active over the disease course [Kennedy *et al.*, 1993], in both genders [Pimentel-Santos *et al.*, 2012c]. This observation has clinical implications, and influence therapeutic decisions even on longstanding AS cases. No analysis in this subject was performed in this cross-sectional study. However, comprehensive assessment of different therapeutic approaches can be made. Globally, there is widespread use of nonsteroidal anti-

inflammatory drugs (NSAIDs) as seen in Portugal (79.1%). Unfortunately, due to the nature on data collection it was not possible to analyze the influence of "daily" *vs.* "on demand" therapy in disease severity. There is some evidence in the literature suggesting that chronic systematic daily intake may have a beneficial effect at structural level [Wanders *et al.*, 2005a]. Additional studies are needed in this field. Interestingly corticosteroids prescription is high (17.6% in total; 12.5% in axial form). Similar results were reported in a previous publication involving SpA (predominantly AS) Portuguese patients [Sousa *et al.*, 2008]. The proportion of prednisone prescription were higher in females (16.6% males *vs.* 20.3% females, $p>0.05$) than in males as it happens in PSOAS cohort (3.6% males *vs.*10% females, $p<0.05$) - another cross-sectional study, performed in North America, involving patients over 20 years of disease duration, [Lee *et al.*, 2007]. The prescription of Sulphasalazine (SSZ) is also high (overall prescription of 36.9%, in monotherapy 30.9%) even when considering axial disease presentation exclusively (32.1%). There weren't any significant differences between genders (37% in males *vs.* 36% in females, $p>0.05$), as in the PSOAS cohort (6.6% in males *vs* 11% in females, $p>0.05$) [Lee *et al.*, 2007], despite of remarkable high proportion of patients taking SSZ in Portugal. This seems to be related with the emerging data supporting SSZ use in early inflammatory back pain and not only in peripheral arthritis involvement [Goh & Samanta, 2009]. The high corticoids and SSZ prescription (even in axial forms of the disease) are probably related to the perception by the Portuguese Rheumatologists of high disease activity. Contrarily, and paradoxically, anti-TNFα prescription was generally low (22%). In fact, the need for anti-TNFα therapy was previously estimated as 30-49% [Barkham *et al.*, 2005; Pham *et al.*, 2006]. The high frequency of co-morbidities, functional impairment, and active disease despite the optimal use of NSAID therapy were the main reasons appointed to start anti-TNFα therapy in Belgium [Vander Cruyssen *et al.*, 2007]. The study performed in Portugal didn't

allow patients characterization when anti-TNFα therapy was started. However, there is indirect evidence - positive correlation between BASMI and anti-TNFα therapy,that biological treatments began late in the course of the disease. The low anti-TNFα associated with high corticoids and SSZ prescriptions may indirectly indicate some economic issues, or alternatively some concerns about using drugs recently introduced in the market. This aspect could explain, at least partially, the lack of adequate control of the disease.

Finally, this study aimed to examine the socio-economic impact of AS and the influence of gender in this context. A detailed assessment of this topic has not been undertaking so far. The available data in the literature is scarce and are limited to studies conducted in the USA [Singh & Strand, 2009] and Taiwan [Chen *et al.*, 2011]. Both studies were based on administrative database, but report different conclusions. The USA study reports that gender didn't influence healthcare utilization in AS patients. The Taiwanese study reports that men had increased risk of high cumulative outpatient visits and a trend for high frequency of hospitalization. Methodological aspects may explain again these differences. The USA study investigated both AS and non-AS related healthcare utilization for only one year and has just examined the gender factor as it was based in a veteran population. The Taiwanese only focused in AS related healthcare utilization over 12 years. According to data collection it is possible to anticipate the limitations of the study performed in Portuguese population. It was hospital-based (tertiary rheumatology practices), so despite being a representative population, it may have different socio-demographic characteristics in comparison to community-based studies. Another restricting factor concerns data collection - "patient-reported". It is well known the inaccuracy of patient-reports, with under-reporting juxtaposed to a higher number of visits, compared with medical record ascertainment [Roberts *et al.*, 1996]. Nevertheless, in

terms of society and health politics it is relevant to analyze the Portuguese data in order to show the differences in health care systems and country settings, which underlie the obtained disparity in different countries. Furthermore this study may contribute to identify the predictors for higher cumulative healthcare services utilization in both genders.

In summary, this data analysis point out ethnic differences and suggest that the AS phenotypic pattern might be different according to the gender. The underlying pathogenesis of these differences is still unknown, warranting further investigation of the topic. In this field, genetic studies represent an important tool to clarify the mechanisms involved in AS.

3.2. LESSONS FROM DNA TECHNOLOGIES IN ANKYLOSING SPONDYLITIS

There is a great need for further research on disease etiopathogenesis and for development of new therapies in AS. Recent advances in the genetics of AS provide a huge amount of information that needs validation. The present work aimed at contributing to this field.

3.2.1. GWAS and Ankylosing Spondylitis

The first-large scale SNP association study in AS, was the WTCCC/TASC study; it involved the genotyping of 14,436 non-synonymous SNPs (nsSNP) and 897 major histocompatibility complex (MHC) SNPs, in 1000 cases and 1500 controls [WTCCC/TASC, 2007]. As this study only addressed the genes' coding regions, a large proportion of the overall genetic diversity remained uncovered [WTCCC/TASC, 2007]. Two major genes, Interleukin 23 Receptor *(IL23R)* and Endoplasmic Reticulum Aminopeptidase 1 *(ERAP1)*, were identified and held responsible, for 9% and 26%, respectively, of the population attributable risk of AS. These findings changed the focus of immunological research in AS to TH17 lymphocytes, as these cells express *IL23R* and hypothesized the interaction between *ERAP1* and *HLA-B27* in peptide presentation. However, most associations may involve non-coding sequences.

The TASC study conducted in 2,053 unrelated AS cases among people of European descent and 5,140 ethnically matched controls, with replication in an independent cohort of 898 AS cases and 1,518 controls [TASC, 2010], has led to interesting results. In addition to the strong association with the major histocompatibility complex, association with SNPs in two gene deserts at 2p15 and 21q22, as well as in Anthrax Toxin Receptor 2 *(ANTXR2)* and Interleukin 1 Receptor, Type II *(IL1R2)* genes were identified. The previously reported associations of

IL23R and *ERAP1* to disease were also replicated [TASC, 2010]. Furthermore, Caspase recruitment domain family, member 9 *(CARD9),* and Tumor necrosis factor receptor superfamily, member 1A - Associated via Death Domain *(TRADD* genes provide suggestive evidence of association with AS in two follow-up studies [Pointon *et al.*, 2010a, 2010b].

In the recently published TASC/WTCCC2 study, Runt-related transcription factor 3 *(RUNX3),* Tumor necrosis factor receptor superfamily, member 1A *(TNFRSF1A),* Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40) *(IL12B),* Prostaglandin E Receptor 4 (subtype EP4) *(PTGER4),* TBK1 binding protein 1 *(TBKBP1),* and again *ANTXR2* and *CARD9* regions showed definite association with AS [Evans *et al.*, 2011]. A summary of all involved genes is presented in Table 4 and Figure 2 (where a spatial genes' distribution in chromosomes is presented).

Recent findings suggest that Tumor Necrosis Factor (ligand) Superfamily, Member 15, *(TNFSF15)* [Zinovieva, 2009] and/or Tumor Necrosis Factor (ligand) Superfamily, Member 8 *(TNFSF8)* [Zinovieva, 2011] may be involved in AS. Neither of these genes was associated with disease in the previous TASC/WTCCC studies [WTCCC/TASC, 2007; TASC, 2010, Evans *et al.*, 2011]. As mentioned earlier, a detailed analysis of the genes studied in the Portuguese population will be discussed.

Table 4: Summary of genetic associations with AS.

Gene	*Locus*	Studies	Definite/Suggestive	Contribution to AS (%)	Mechanism
HLA-B27	6q21	Brewerton,1973 Schlosstein,1973	Definite	23.3	Presentation peptides to T-cells
Gene deserts	2p15	TASC,2010	Definite	0.543	Unknown
ERAP1	5q15	WTCCC/TASC,2007	Definite	0.34	Peptide trimming to HLA class I presentation
IL23R	1p31	WTCCC/TASC,2007	Definite	0.31	Differentiation of IL23R expressing cells
KIF21B	1q32	Danoy P,2010	Definite	0.25	Unknown
RUNX3	1p36	Evans DM,2011	Definite	0.12	Reduction in CD8 lymphocyte counts
IL1R2	2q11	TASC,2010	Definite	0.12	Influence on IL-1 response
IL12B	5q32	Danoy P,2010	Definite	0.11	Differentiation of IL23R expressing cells
TNFR1	12p13	TASC,2010 Davidson,2010	Definite	0.075	TNF signaling
ANTXR2	4q21	Evans DM,2011	Definite	0.054	Unknown
TBKBP1	17q32	Evans DM ,2011	Definite	0.054	TNF signaling
PTGER4	5p13	Evans DM ,2011	Definite	0.052	Induction of IL23 expression/Differentiation of IL23R expressing cells/ Bone anabolism.
Gene deserts	21q22	TASC,2010	Definite	0.035	Unknown
CARD9	9q34	TASC, 2010 Pointon, 2010	Definite	0.034	Th17 activation after β-glucan exposure
TNFSF15	9q32	Zinovieva, 2009	Suggestive	-	Th17 activation/differentiation
TRADD	16q22	Evans DM ,2011	Suggestive	-	TNF signaling
STAT3	17q21	Danoy P,2010 Davidson,2010	Suggestive	-	Differentiation of IL23R expressing cells
KIR3DS1	19q13	Zvyagin, 2010	Suggestive	-	NK cell inhibition/activation
TNFSF8	9q33	Zinovieva, 2011	Suggestive	-	CD30L/CD30 signaling

Level of confidence – 'Confirmed' indicates P<5x10-7 in one study, with replication in a second cohort. 'Suggestive' indicates 10-5<P<5x10-7 in one study, or P<5x10-7 but no replication. (Adapted from Brown, 2011).

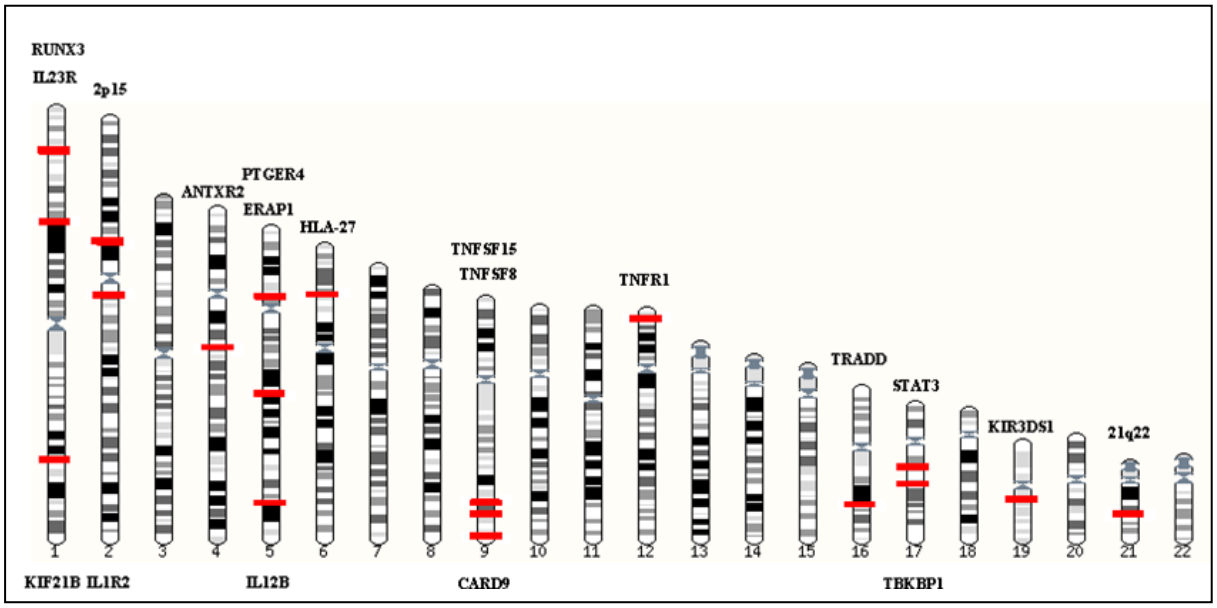

Figure 2 - Chromosomal locations of genes implicated in AS susceptibility in GWAS. (Adapted from Santos *et al.*, 2007)

3.2.2. MHC genes

3.2.2.1. *HLA-B27*

The MHC on chromosome 6p is the main locus strongly linked and associated with AS, although, as determined forty years ago, the majority of genetic associations are driven by *HLA-B27* allele [Schlosstein *et al.*, 1973; Brewerton *et al.*, 1973]. *HLA-B27* is present in more than 80% of white European AS cases, compared with approximately 8% of healthy controls [Brown *et al.*, 1996]. Our study has shown that 80.3% of AS Portuguese patients are *HLA-B27* positive [Pimentel-Santos *et al.*, non-published data]. The worldwide distribution of *HLA-B27* varies considerably, as previously commented, and there is a clear decreasing gradient in its frequency from north to south [Khan, 1995; Gonzalez-Roces *et al.*, 1997; Blanco-Gelaz, 2001]. In general, the prevalence of AS is strongly correlated with *HLA-B27*

distribution and the strength of their association varies among different ethnic populations [Blanco-Gelaz, 2001]. However, in the general population only 1-5% of *HLA-B27* positive subjects develop AS [van der Linden *et al.*, 1984b; Gran *et al*., 1985a; Braun *et al.*, 1998]; the proportion increases to 20% in positive relatives of AS patients [van der Linden *et al*., 1984b]. Family or twin studies have estimated a range from 16 to 50% for *HLA-B27* contribution to AS [Rubin, 1994; Brown *et al*., 1997; Brown *et al*., 1998a] while the whole effect of the MHC is approximately 50% [Brown *et al*., 1998; Laval *et al*., 2001; Zhang G *et al*., 2004]. Independent twin studies have shown that heritability of susceptibility to AS is over 90% [Brown *et al*., 1997; Pedersen, 2006], and it is likely that other genetic factors (inside and outside the MHC) may influence susceptibility to AS.

Despite extensive research over the past decades, the exact mechanism underlying the effect of *HLA-B27* on AS susceptibility has not yet been established. Several theories have tried to explain possible underlying mechanisms and include:

a) Canonical mechanisms:

- The “arthritogenic peptide” theory explains the association of *HLA-B27* with AS in terms of the function of HLA-Class I molecules in peptide presentation to CD8+ T lymphocytes [Kuon *et al*., 2001; Boyle & Gaston, 2003; Sims *et al*., 2004].

- The “molecular mimicry” theory focused in cross-reactivity between host self' antigens and specific disease triggering microorganisms’, presented by *HLA-B27* or HLA-class II presentation to CD4+ T lymphocytes [Schwimmbeck & Oldstone, 1988; Benjamin & Parham, 1992; Boyle *et al*., 2001; 2004a; 2004b; Boyle & Gaston, 2003].

b) Non-canonical mechanisms:

- The induction of endoplasmic reticulum stress due to the propensity of HLA-B27 to fold slowly and inefficiently [Mear *et al.*, 1999; Martin, 2002].

- The occurrence of abnormal forms of HLA-B27 such as homodimers, leading to aberrant immunological reactions [Allen *et al*., 1999].

- The release of β2-microglobulin from a subpopulation of cell surface - expressed HLA-B27 molecules leads to its deposition within synovia and to the initiation of an inflammatory process [Uchanska-Ziegler & Ziegler, 2003].

- The nearby "linked gene" theory, that proposes *HLA-B27* as a marker for a nearby true disease susceptibility locus [Nijenhuis, 1977].

Until now (July 2011), eighty-one (*HLA-B*2701-B*2782; B*2722* has been removed because it resulted from a sequencing error) molecular *HLA-B27* subtypes have been assigned, and many other synonymous polymorphisms were reported in B27 alleles (in The Antony Nolan Trust database, http://hla.alleles.org/alleles/class1.html). Most subtypes have only been reported in a few individuals and, therefore, it is not possible to know whether they are associated with AS [Reveille & Maganti, 2009]. However, there are reports of AS or SpA association with *HLA-B*2701* [MacLean *et al.*, 1993], *B*2702* [MacLean *et al.*, 1993], *B*2703* [Armas *et al.*, 1999], *B*2704* [López-Larrea *et al.*, 1995a], *B*2705* [MacLean *et al.*, 1993], *B*2706* [Gonzalez-Roces *et al.*, 1997], *B*2707* [Armas *et al.*, 1999], *B*2708* [Armas *et al.*, 1999], *B*2710* [García *et al.*, 1998], *B*2714* [García-Fernández *et al.*, 2001], *B*2715* [García-Fernández *et al.*, 2001] and *B*2719* [Tamouza *et al.*, 2001]. Actually, there is

evidence that distinct subtypes are differentially associated with AS; two subtypes, *B*2706* and *B*2709*, found in southeast Asia and Sardinia, respectively, have shown significantly reduced association with AS [D'Amato *et al.*, 1995; Lopez-Larrea *et al.*, 1995a]. In our Portuguese population, only *B*2705* and *B*2702* were identified in AS patients (n=51); in controls (n=16), *B*2707* was also identified [Pimentel-Santos *et al.*, 2007].

3.2.2.2. Other MHC genes

Several MHC genes have been implicated in AS susceptibility and/or its clinical manifestations. In fact, about 10% of Caucasian patients with AS are *HLA-B27* negative [Schlosstein *et al.*, 1973; Brewerton *et al.*, 1973]. In Portugal [Pimentel-Santos *et al.*, 2012c] and Greece [Alamanos *et al.*, 2004] the proportion of *HLA-B27* negative is about 20% and in Turkey is even high (about 30%) [Gunal *et al.*, 2008]. The association of *HLA-B60* (*B*40*) in both, *HLA-B27* positive and negative individuals, has been largely replicated and confirmed in groups such as Taiwanese, Chinese and Europeans [Robinson *et al.*, 1989; Rubin *et al.*, 1994; Brown *et al.*, 1996; Wei *et al.*, 2004]. Others *HLA-B* associations have been reported such as *HLA-B38* and *HLA-B39* in Japanese [Yamaguchi *et al.*, 1995], and *HLA-B35* and *HLA-B62* in *HLA-B27* negative Caucasians patients [Wagener *et al.*, 1984]. However, these associations have not been independently confirmed in additional studies. In our Portuguese population only *HLA-B8* was significantly increased in AS patients *versus* controls ($p=0.003$, $OR=3.97$, $1.47<CI<10.8$). However, after Bonferroni correction no statistically significant differences were found between both groups.

Other MHC class I genes have been tentatively associated with AS, in particular *MICA*, but the association was better explained by LD with *HLA-B27* [Yabuki *et al.*, 1999; Martinez-Borra *et al.*, 2000; Ricci-Vitiani *et al.*, 2000; Singal *et al.*, 2001;].

MHC class II alleles have been also implicated in AS susceptibility, such as *HLA-DRB1*01*, (independently of *HLA-B27*) in the British and Mexican population [Brown *et al.*, 1998b; Vargas-Alarcon *et al.*, 2002a], HLA-*DRB1*08* in British [Brown *et al.*, 1998b] and *HLA-DQ*02* in Spanish patients [Sanmartí *et al.*, 1987]. In addition, *HLA-DRB1*08* seems to confer susceptibility to AAU [Monowarul Islam *et al.*, 1995]. Transporter 1, ATP-binding cassette, sub-family B *(TAP1)* and Transporter 2, ATP-binding cassette, sub-family B *(TAP2)*, which is located within MHC class II region, was proposed as AS associated but in a Spanish study this effect was not seen [Fraile *et al.*, 2000]. The Low molecular weight proteosome 2 (*LMP2)* was also implicated in juvenile AS patients from Mexico [Vargas-Alarcón *et al.*, 2004] and Norway [Ploski *et al.*, 1995] and it also seems to increase risk for AAU in AS patients from Canada [Maksymowych *et al.*, 1997]. Moreover, *LMP7* alleles have been reported to be associated with AS in Spanish patients [Fraile *et al.*, 1998a]. In our population no association was established between HLA-DR and HLA-DQ alleles studied and AS. In contrast, several associations of these alleles with phenotypic characteristics were identified, in HLA-B27 positives patients. DRB1*08 allele seems to be linked to increased risk of AAU, whereas DQB1*04 appears to provide consistent protection in terms of disease activity (BASDAI), functional (BASFI), metrological (BASMI), and radiological severity (mSASSS).

Several studies have addressed the association of the MHC class III region and AS. Tumor necrosis factor alpha (TNF-α), a macrophage derived cytokine, has been implicated in the pathogenesis and development of AS [Tracey & Cerami, 1993]. In addition, TNF-α blockers

have proven to be effective in improving AS manifestations, which suggests that TNF is at least as important for inflammatory process in AS as it is in other rheumatic diseases [Davis *et al*., 2003; van der Heijde *et al*., 2005, 2006]. *TNF* gene has 12 *kb* and is located in MHC class III region, between class I and II regions and adjacent to *HLA-B* locus [Glossop *et al*., 2003]. Several SNPs are common in *TNF* gene promoter [Allen, 1999]. These genetic variants could affect the binding of transcription factors, which might in turn influence the promoter's activity and, ultimately, mRNA and protein levels [Bayley *et al*., 2004]. A number of studies have been focused on the promoter position −308 (G/A) and −238 (G/A) of the *TNF* gene [Verjans *et al.*, 1994; Fraile *et al.*, 1998b; Hohler *et al.*, 1998; Kaijzel *et al.*, 1999; McGarry *et al.*, 1999; Martinez- Borra *et al.*, 2000; Milicic *et al.*, 2000; Gonzalez *et al.*, 2001; Vargas-Alarcon *et al.*, 2006; Shiau *et al.*, 2007; Lu *et al.*, 2008; Chatzikyriakidou *et al.*, 2009; Nicknam *et al.*, 2009; Sousa *et al.*, 2009]. However, there were inconsistent results among these studies that might be due to sample size, insufficient statistical power, clinical heterogeneity, and ethnic differences.

In Portuguese population, the low frequency of A allele at -238 position in AS – as compared to control and independently of *HLA-B27* - suggested a protective role for this allele [Sousa *et al.*, 2009]. Similar results were obtained in populations issued from Germany [Hohler *et al.*, 1998] and Taiwan [Shiau *et al.*, 2007]. On the contrary, in our study no association has been detected between polymorphisms at position -308 and AS. Repercussion of these SNPs on disease prognosis has been less studied; nevertheless, in national patients, -308 GA/AA and -238 GA/AA genotypes were associated with a later disease onset and high erythrocyte sedimentation rate (ESR) values, respectively [Sousa *et al.*, 2009]. Taken together, these

results suggest that *TNF* gene promoter SNPs at -238 and -308 positions might have a small influence on AS susceptibility and disease prognosis.

Recently, two meta-analysis were performed to evaluate the association of *TNF* promoter polymorphisms with AS susceptibility [Lee & Song, 2009; Li *et al.*, 2010]. No association was found between *TNF* promoter -238 and -308 polymorphisms and AS susceptibility in either the overall population or the HLA-B27 positive subjects. Although meta-analysis is a powerful method to overcome the problem of small sample size and inadequate statistical power frequently encountered in published studies [Egger *et al.*, 1997], some limitations existed in both of the above mentioned publications; namely, a small proportion of the AA genotype for both positions -238 and -308 in the *TNF* promoter; differences in gene distribution between studies; and publication biases. Moreover, only two studies investigated the role of these polymorphisms in *HLA-B27* negative AS patients [Martinez-Borra *et al.*, 2000; Gonzalez *et al.*, 2001] and in both no definitive conclusions could be taken.

Heat shock protein *(HSP70)* is another factor involved in immune response, coded by a gene located in MHC class III region, in the vicinity of HLA-B locus. HSP70 gene has been investigated regarding its potential influence on susceptibility to AS. There was a significant association of *HSP70-2* and *HSP70-hom* alleles with SpA in Mexicans, in both *HLA-B27* positive and *HLA-B27* negative individuals [Vargas-Alarcón *et al.*, 2002b]. However, conflicting results were obtained in other populations; no association between *HSP70* gene polymorphism and AS was identified in Chinese Han [Lin *et al.*, 2004, 2007], Finish [Westman *et al.*, 1994], nor Spanish [Fraile *et al.*, 1998c] populations. For this reason, this gene's role must still be enlighted in further future studies. There aren't any available data in this regard in Portuguese population.

In summary, despite a broad plethora of published data, results must be interpreted with caution as there is still no agreement among different studies. In addition, WTCCC2/TASC study [Evans *et al.*, 2011] didn't find any significant MHC associations except for HLA-B locus.

Extreme allelic diversity in this region and extensive LD makes it difficult to genotype, analyze, and identify allele(s) directly associated with pathology. This high diversity adds to the possibility that AS might be associated with more than one MHC gene and explains why this region is so hard to study [Brown, 2010]. HLA genes are organized in haplotypes which renders dissection of individual genes very difficult; it is, thus, also very hard to spot out "predisposing" haplotypes. However, it is of utmost importance to identify haplotypes that might be linked to disease, either by being causative variants of disease or by influencing the disease's phenotypic characteristics [Fiorillo *et al.*, 2003]. This was the underlying reason to have performed extensive haplotypes analysis in this work.

3.2.3. MHC haplotypes

There are several scattered studies in the literature based on MHC-haplotype analysis that report conflicting results that hinder clear conclusions. The main reason for differences might be related, once again, to under-powered statistical analysis, heterogeneous groups of patients, or different methodologies used.

In our Portuguese population, extended HLA haplotypes in B27 positive patients and controls were estimated by Expectation Maximization algorithm using the Arlequin v3.11 software. To the author's knowledge, it was the first study where extended haplotypes were evaluated.

*A*02/B*27/Cw*02/DRB1*01/DQB1*05* haplotype has been identified exclusively in AS patients and it thus seems to confer susceptibility to the disease. In addition, *A*02/B*27/Cw*01/DRB1*08/DQB1*04* haplotype seems to confer protection in terms of disease activity (BASDAI), functional (BASFI), and radiological repercussion (mSASSS). [Pimentel-Santos *et al.*, non-published data].

Previous studies are partial evaluations within MHC. In Sardinia two different haplotypes were described as conferring susceptibility to AS, the *A*02/B*2705/Cw*02/DR*02* and *A*02/B*27/Cw*02/DR*16* [Contu *et al.*, 1992; Fiorillo *et al.*, 2003]. In Tunisian [Kchir *et al.*, 2010] and in British [Sims *et al.*, 2007] populations different haplotypes were also described, *B*27/DRB1*11/DQB1*03* and *B*27/DRB1*07*, respectively. Data regarding haplotypes involving HLA-B/-Cw are scarce, but *B*27/Cw*01* and *B*27/Cw*02* were described in association with AS in some Caucasian populations [González-Roces *et al.*, 1994; López-Larrea *et al.*, 1995b; Ben Radhia *et al.*, 2008].

There is a lack of information in the literature regarding association between MHC-haplotypes and clinical features of disease. Our Portuguese Study may be a contribution in this field. Complementary information was gained from one small cohort study of *HLA-B27* positive Taiwanese AS patients. The haplotypes *A*33/B*58/Cw*10*, *A*33/B*58/Cw*10/DR*13*, and *A*33/B*58/Cw*10/DR*17* showed a strong negative association with bamboo spine [Lu *et al.*, 2009].

This particular field of research remains opens to further understanding and therefore presents an excellent opportunity for future studies.

3.2.4. Non-MHC genes

3.2.4.1. *ERAP1*

The WTCCC/TASC nsSNP study in 2007 led to the identification of *ERAP1* as the first non-MHC gene definitively associated to AS [WTCCC/TASC, 2007]. The association was confirmed in Portuguese patients according to our data [Pimentel-Santos *et al.*, 2009] and thereafter in a wide variety of ethnic groups, including, Canadians [Maksymowych *et al.*, 2009], Han Chinese [Davidson *et al.*, 2009; Li *et al.*, 2011], Koreans [Choi *et al.*, 2010], and Hungarians [Pazar *et al.*, 2010]. In Portuguese population, the strongest associated *ERAP1* polymorphism was rs27044. However in our data no association was found with marker rs2303138 lying in *LNPEP*, which supports the hypothesis that at least a component of the association observed between this SNP and AS, previously reported in British Caucasians, is due to LD with *ERAP1* polymorphisms [Pimentel-Santos *et al.*, 2009]. Recently, a meta-analysis was performed to assess the strength of association between *ERAP1* polymorphisms and AS risk, involving nine case-control studies (total of 8,530 AS patients and 12,449 controls) [Chen *et al.*, 2011]. This meta-analysis further confirmed that *ERAP1* polymorphisms may play a significant role in AS susceptibility. Except for rs27434, a meaningful correlation was detected in rs27044, rs17482078, rs10050860, rs30187, rs2287987, and rs27037.

Considering the imputed and genotyped data across *ERAP1* performed in our study, an association was seen between SNPs rs26509 and rs190298, an interval of 84 *kb*. In the WTCCC2/TASC, association is clearly localized in a more restrict block of 4.6 *kb* region

between rs27529 and rs469758. Recently, the primary associated variant(s) in *ERAP1*, the major allele of rs30187, and the minor allele of rs10050860 were shown to confer a protective effect against AS [Evans *et al.*, 2011]. This association is restricted to *HLA-B27* positive cases [Evans *et al.*, 2011].

No significant association has been reported between *ERAP1* and IBD [WTCCC/TASC, 2007], although there is an association with the enthesitis related arthritis subtype of juvenile idiopathic arthritis [Hinks *et al.*, 2011] and psoriasis [Genetic Analysis of Psoriasis Consortium & the WTCCC2, 2010; Sun LD *et al.*, 2010]. Interestingly, it appears that *ERAP1* variants only influenced psoriasis susceptibility in individuals carrying the HLA-C risk allele; this fact brings to attention the same functional interaction that exists between *HLA-Cw6* and *ERAP1* and between *HLA-B27* and *ERAP1*. In addition, there are moderate levels of association with type 1 diabetes [Fung *et al.*, 2009] and cervical cancer [Mehta *et al.*, 2007, 2008, 2009]. ERAP1 is an aminopeptidase with ubiquitous tissue distribution that is expressed in the endoplasmic reticulum. ERAP1 is a zinc-metallopeptidase with typical H-E-X-X-H-(X)(18)-E zinc binding and G-A-M-E-N motifs characteristic of the members of the gluzincin protease family. These structures reveal extensive domain movements, including an active site closure, as well as three different open conformations, thus providing insights into the catalytic cycle. A K(528)R mutant strongly associated with AS in GWAS studies shows significantly altered peptide processing characteristics, which are possibly related to impaired interdomain interactions [Kochan *et al.*, 2011]. The exact mechanism by which *ERAP1* influences AS susceptibility is not clear. Two hypotheses have been considered:

a) To trim peptides, acting as a molecular ruler, down to nine amino acids in length prior to loading into nascent HLA class I molecules [Chang *et al.*, 2005; Saveanu *et al.*,

2005]. *In vitro*, the use of recombinant ERAP1 showed that rs31087 and rs17482078 were associated with markedly reduced peptide trimming, whereas rs10050860 and other non-synonymous variants were neutral. However, as previously commented, polymorphisms of *ERAP1* only affect AS risk in *HLA-B27* positive individuals [Evans *et al.*, 2011]. These findings are extremely important to determine the mechanism of *HLA-B27* and AS association.

b) Through its potential role in cleaving pro-inflammatory cytokine receptors from the cell wall, including TNFR1 [Cui *et al.*, 2002], IL-1R2 [Cui *et al.*, 2003a] and IL-6R [Cui *et al.*, 2003b]. If a definitive association of *IL1R2* and a suggestive association of *TNFR1* with AS were demonstrated, [TASC, 2010], as well as an increase of IL6 plasmatic levels [Keller *et al.*, 2003], the lack of differences in *ERAP1* expression between AS and controls, in peripheral blood [TASC, 2010], would show this function does not have a significant physiological role. To reinforce this finding, the ability of ERAP1 to cleave TNFR1 and IL-6R from cell surfaces was tested by measuring these receptors in cell culture supernatants (from single-cell suspensions prepared from *Erap1*−/− and C57BL/6 control mouse spleens). No difference was observed in the levels of these receptors over time, indicating that *ERAP1* does not have a major influence on cytokine receptor cleavage, at least in mice [Evans *et al.*, 2011].

Further studies in different ethnic groups and new functional studies would bring new insight into the mechanisms involved in AS.

3.2.4.2. *IL23R*

The WTCCC/TASC nsSNP study in 2007 also identified the association of *IL23R* and AS [WTCCC/TASC, 2007]. The association was confirmed in an independent North American cohort, and thereafter replicated in a wide variety of ethnic groups, including Spanish [Rueda *et al.*, 2008], Canadian [Rahman *et al.*, 2008], Portuguese [Pimentel-Santos *et al.*, 2009] and again in English [Karaderi *et al.*, 2009] population. The studies in East Asians did not show any association between the gene and AS in Han Chinese [Davidson *et al.*, 2009] nor in Koreans [Sung *et al.*, 2009] or in Japanese with Crohn's disease [Yamazaki *et al.*, 2007], or in Chinese with psoriasis [Liu *et al.*, 2008].

The peak association in our Portuguese cohort was seen with rs1004819, and this finding is different from what was found from American, Canadian, Spanish and British data sets, although the minor allele frequencies (MAF) observed for SNPs in Portuguese patients were similar to those reported in any other populations. Furthermore, the association we observed in the Portuguese population had a similar magnitude of effect when compared to the one described in those other populations: the attributable risk for rs1004819 is very similar to the one reported for the most strongly associated SNP (rs11209032) in the British/North American populations [WTCCC/TASC, 2007]. In order to identify the primary SNP variant associated to AS, the imputed SNP data showed that a broad region of *IL23R* is associated with AS in Portuguese patients. Association peak range between the SNPs rs10889667 and rs11465817, an interval of 66 *kb*, with a rapid reduction in strength of association outside that region. It suggests that the primary associated variant(s) lies in that interval [Pimentel-Santos *et al.*, 2009]. In TASC study an imputation analysis was performed to narrow the likely region

of association. The strongest association was seen with rs11465817, which lies in intron 9, and with rs11209026 (which codes for Gln381Arg substitution), in exon 9. Curiously, our Portuguese study did not demonstrate any protective effect against AS for the Arg381Gln SNP (rs11209026) in the *IL23R* gene, which is consistent with the Alberta Canadian population. This apparent lack of concordance may be due to the different ethnical backgrounds studied, or represent a type II error, given the modest power of the Portuguese study to detect association with this rare, protective SNP. Recently, two definite SNPs in *IL23R*, rs11209026 and rs11209032 were identified in association with AS [Evans *et al.*, 2011]. Interestingly, rs11209026 was not polymorphic in the Asian population, which may explain the lack of association between common variants in *IL23R* and AS in this population.

IL23R is also known to be associated with IBD [Duerr *et al.*, 2006; Dubinsky *et al.*, 2007], psoriasis [Cargill *et al.*, 2007; Wu *et al.*, 2010], and psoriatic arthritis [Rahman *et al.*, 2008; Bowes & Barton *et al.*, 2010] including the juvenile-onset psoriatic arthritis [Hinks *et al.*, 2011], which partly explains the frequent coexistence of these pathologies in individual cases and families.

The *IL23R* association with AS was the first suggestion that TH17 lymphocyte pathway may be involved in disease pathogenesis. Data supporting an increase in TH17 lymphocytes numbers in AS [Jandus *et al.*, 2008], and an increase of serum IL17 levels [Wendling *et al.*, 2007], pinpoint a role for these cells. Moreover, other related genes in the IL23R pathway have also been implicated in AS, such as **S**ignal Transducer and Activator of Transcription 3 (acute-phase response factor) *(STAT3)* [TASC, 2010], Janus kinase 2 *(JAK2)* [TASC, 2010], *IL12B* [Danoy *et al.*, 2010], *CARD9* [TASC, 2010; Pointon *et al.*, 2010a], and *PTGER4* [Evans *et al.*, 2011]. STAT3 is a key transcription factor in IL23R signaling; *JAK2* encodes

the partner of STAT3 in this pathway. Both of them are also associated with IBD [Barrett *et al.*, 2008]. *IL12B* encodes IL-12p40, a component of the heterodimeric cytokines, IL-12 and IL-23. *CARD9* encodes a component of the Dectin-1 involved in innate immunity, by recognizing β-glucan, a component of fungal and some gram-negative bacteria cell wall which promote the activation of TH1 and TH17 lymphocytes, thereby linking the innate and adaptive immune systems [LeibundGut-Landmann *et al.*, 2007]. *PTGER4* encodes the PGE2EP4 receptor, one of the four known PG2E receptors (named EP1-4), which have several effects and are expressed in several tissues. In response to β glucan recognition, PGE2 is produced, and the interaction with PGE2EP4R stimulates dendritic cell production of IL-23 leading to TH17 activation [Chen *et al.*, 2010]. In collagen-induced arthritis the inhibition of *PGE2EP4*, in both prophylactic and therapeutically models, has shown to attenuate the inflammatory process [Chen *et al.*, 2010]. Another interesting point is the link between inflammation and new bone formation, promoted by *PGE2EP4*, which seems relevant in AS pathogenesis. Repetitive mechanical stress has been shown to up regulate PGE2 production and in turn new bone formation at entheses [Zhang & Wang, 2010]. Therefore there is some evidence that non-steroidal anti-inflammatory drugs, which inhibit PGE2 production, may reduce bone formation in AS [Wanders *et al.*, 2005a, 2005b].

All this knowledge changed the way of thinking about AS pathogenesis and promoted the development of new potential therapies. Interesting results have been documented with Ustekinumab, an anti-IL12p40 antibody targeting the shared IL-23/IL-12 subunit, in psoriasis [Kauffman *et al.*, 2004; Toichi *et al.*, 2006], psoriatic arthritis [Kavanaugh *et al.*, 2010; Zhu *et al.*, 2010], and Crohn's disease [Mannon *et al.*, 2004; Fuss *et al.*, 2006]; no data is available yet to asses the effect of Ustekinumab in AS. Secukinumab (AIN457), another fully human

monoclonal antibody targeting IL-17A, demonstrated preliminary efficacy in psoriasis [Hueber *et al.*, 2010; Kurzeja *et al.*, 2011] and in non-infectious AAU [Hueber *et al.*, 2010]. Preliminary data has shown that this agent may be useful in the treatment of moderate to severe active AS [Baeten *et al.*, ACR 2010].

3.2.4.3. *ANKH*

Conflicting results regarding *ANKH* association with AS have been published. Our study and, another previous one, of *ANKH* showed no association with susceptibility to AS [Timms *et al.*, 2003; Pimentel-Santos *et al.*, 2012a]. However, weak positive findings have also been reported by some authors [Furuichi *et al.*, 2008], and it has been suggested that this association may be more strongly observed in women [Tsui *et al.*, 2005]. *ANKH* variants studied in our Portuguese population did not seem to exert any influence in either men or women [Pimentel-Santos *et al.*, 2012a]. Furthermore, no association has been identified with this gene in GWAS in AS until now, which suggests *ANKH* may not be a major determinant of susceptibility to AS [Burton *et al.*, 2007; Evans *et al.*, 2011; TASC, 2010].

ANK is a multipass transmembrane protein encoded by *ANKH* gene that regulates cellular inorganic pyrophosphate (PPi) export across the plasma membrane to the extracellular compartment. The interest in this gene is therefore related to the potential effect in bone metabolism. A loss-of-function mutation in the homologous gene, *ank*, in a mice model, is associated with the development of severe ectopic mineralization and skeletal ankylosis, resembling AS [Ho *et al.*, 2000]. Humans with gain-of-expression polymorphisms in this gene develop calcium pyrophosphate chondrocalcinosis [Williams *et al.*, 2002; Zhang *et al.*,

2005], whereas loss-of-function polymorphisms cause excess hydroxyapatite deposition as seen in Jackson's craniometaphyseal dysplasia [Numberg *et al.*, 2001; Reichenberger *et al.*, 2001]. For all the above-mentioned aspects, it was hypothesized that *ANKH* polymorphisms may contribute to spinal ossification in AS; such an effect would lead to changes in BASMI and mSASSS scores. However *ANKH* variants studied in our research didn't seem to have any significant association with these scores [Pimentel-Santos *et al.*, 2012a]. It seems, thus, that *ANKH* polymorphism is not a major determinant of susceptibility to AS and does not have a major influence on disease severity (as measured by BASMI and mSASSS) - at least in Portuguese population.

Most of published studies lacked power. This might account for conflicting results encountered in literature and further research is needed in order to reach definitive conclusions.

3.2.4.4. *TNFSF8*

A genome-wide linkage screen, performed in multiple cases of SpA families, identified a highly significant linked region on chromosome 9q31-34, named SPA2 [Miceli-Richard *et al.*, 2004]. This locus overlaps one of those previously identified in the first two studies of genome-wide linkage scans in AS [Brown *et al.*, 1998a; Laval *et al.*, 2001]; furthermore, meta-analysis of all published linkage scans also supports a linkage of SPA2 and SpA [Carter *et al.*, 2007]. It was estimated that this locus, comprising about 85 genes, might contain major predisposition gene(s), accounting for 20-25% of the non-MHC genetic susceptibility to SpA

[Breban *et al.*, 2006]. To add, SPA2 represents one of the three regions paralogous to the MHC [Said-Nahal *et al.*, 2002].

Systematic LD mapping of the whole SPA2 region using a dense set of tag-SNPs refine the peak association to an interval with a 6-SNPs haplotype spanning 40 *kb* and located between *C9ORF91* and *TNFSF15;* rs6478105 was the strongest individual associated SNP [Zinovieva *et al.*, 2009]. In our recently published study another significant association with SNP rs3181357 lying in the SPA2 locus was identified in a French cohort and subsequently replicated in an independent combined Belgian and Portuguese cohort [Zinovieva *et al.*, 2011].

TNFSF15 and *TNFSF8* are members of the TNF superfamily of genes that are lying in the SPA2 locus [Franke *et al.*, 2010]. *TNFSF8*, codes for CD30L (CD153), a TNF superfamily ligand expressed on activated CD4+ T cells, antigen-presenting cells and neutrophils, which interacts with CD30 on effectors or memory Th cells. CD30L/CD30 signaling seems to play a role in Th17 cell differentiation *in vitro* and *in vivo* [Sun *et al.*, 2010]. *TNFSF15* has been shown to stimulate TH17 lymphocytes proliferation [Pappu *et al.*, 2008]; in an inflammatory colitis mouse model, TNFSF15 up regulates TH1 and TH17 lymphocytes activity [Takedatsu *et al.*, 2008]. Both, *TNFSF8* [Sun *et al.*, 2008] and *TNFSF15* [Franke *et al.*, 2010; Yamazaki *et al.*, 2005] may have a role in IBD. However, no association was seen with *TNFSF8* neither with *TNFSF15* in others studies performed in AS cases [Burton *et al.*, 2007; TASC, 2010, Evans *et al.*, 2011]. Thus, it is not known if *TNFSF8* or *TNFSF15* SNPs described as associated with SpA are directly implicated in SpA predisposition, either alone or in combination with other polymorphisms located in this same region. Alternatively, it could be

a surrogate for one or several other variant(s) not yet identified, by means of LD. Further studies will be required to clarify all these issues.

3.3. LESSONS FROM GENOMIC PROFILING IN ANKYLOSING SPONDYLITIS

3.3.1. Applications of microarrays in rheumatology/spondyloarthritis

Several microarrays studies have been published looking at SpA. A number of early studies used different tissue sources and smaller microarrays with whole-genome arrays prohibitively expensive [Thomas & Brown, 2010a, 2010b]. The first study in 2002 identified genes more highly expressed in peripheral blood mononuclear cells (PBMC) of patients with spondyloarthropathy (SpA), rheumatoid arthritis (RA) and psoriatic arthritis (PsA), in comparison to normal subjects [Gu *et al.*, 2002a]. A 588-gene microarray was used as a screening tool and the results were validated by reverse transcription-polymerase chain reaction (RT-PCR). A total of 16 genes were identified encoding differentiation markers, cytokines, cytokine/chemokine receptors and signaling and adhesion molecules. An increased expression of Chemokine (C-X-C motif) receptor type 4 (*CXCR4*) and its ligand Stromal cell-derived factor-1 (SDF-1), in synovial fluid mononuclear cells (SFMC), were seen in all three arthritis groups. The conclusion was that the CXCR4/SDF-1 is a potential pro-inflammatory axis for SpA, PsA and RA. However no genes were identified that could discriminate between the different diseases.

In another study gene expression profiles of SFMC from SpA and RA patients were compared with PBMC of healthy controls to evaluate the unfolded protein response (UPR) hypothesis and identify which cytokines/chemokines were being expressed and which cell fractions were involved. An 1176-gene microarray was used and the results were validated by RT-PCR. There was an increase in transcripts encoding Monocyte chemotactic protein-1 (MCP-1),

proteasome subunit C2 and Binding immunoglobulin protein (BiP), which suggest the existence of an UPR. BiP was higher in SpA SFMC compared to RA SFMC and macrophages were identified as the potentially cell type involved [Gu *et al.*, 2002b].

A third study identified a gene expression profile in gut biopsies that could differentiate SpA patients with sub-clinical gut inflammation from SpA patients without gut disease. 2625 differentially expressed sequence tags were initially identified through macroarrays in colon biopsies from Crohn's and SpA patients which were then used to construct a microarray which was sued to screen a further sample cohort. Ninety five expressed sequence tags clustered patients with Crohn's and those with SpA and chronic gut inflammation [Laukens *et al.*, 2006].

This topic of the discussion will be focused on studies using peripheral blood and microarray platforms covering the whole genome. The results seem to be quite heterogeneous reflecting the different methodologies involved, as commented above. Several aspects, summarized in Table 5, may introduce variability and bias in the results, specifically;

a) Patient selection: numbers of patients, the criteria used to classify and include the patients, different degrees of activity/severity of the disease and patients receiving different therapies are examples of heterogeneity that might influence the final results.
b) Cell Source used for analysis: PBMC vs. whole blood or a specific cell subset.
c) Differences in microarray platform technology and data analysis tools.
d) Differences in methodology used regarding validation of candidate biomarkers.

Based on seven papers published since 2007, several pathways relevant to potential SpA pathological processes have been identified. Moreover, potential biomarkers with applications to diagnosis and treatment response prediction in clinical practice were also flagged. Table 5, summarizes the similarities and methodological differences between the studies and reinforces the caution that should be observed when translating these findings to clinical practice. All the knowledge obtained must be interpreted as hypotheses which need validation in future studies.

Table 5: Comparison between published microarray studies in SpA context.

	Subjects	Criteria	Samples	Microarray	Validation
Smith *et al.* 2008	6AS+2uSpA 9HC	mNYC ESSG, Amor	Macrophage	Affymetrix	qPCR
Haroon *et al.* 2010	16AS	mNYC	PBMC	Affymetrix	qPCR
Sharma *et al.* 2009	11uSpA+7uSpA+25HC	Likelihood Score	Whole blood	Affymetrix	Microarrays (2 set)
Duan *et al.* 2010	18AS+18HC 35AS+18HC	mNYC	PBMC	Illumina	qPCR
Gu *et al.* 2009	21AS+28uSpA 23AS+18uSpA 26HC+12RA+5LBP	Calin	PBMC	Illumina	qPCR
Assassi *et al.* 2011	16AS + 14HC+ SLE+SSC 27AS+27HC	mNYC	Whole bood	Illumina	qPCR
Santos *et al.* 2011	18AS+18HC 78AS+78HC	mNYC	Whole blood	Illumina	qPCR

AS: Ankylosing spondylitis; **SpA:** Spondyloarthritis; **HC:** Healthy controls; **RA:** Rheumatoid arthritis; **LBP:** Lumbar back pain; **SLE:** Systemic lupus erythematosus; **SSC**: Systemic Sclerosis; **mNYC:** modified New York criteria; **ESSG:** European Spondyloarthropathy Study Group; **PBMC:** Peripheral blood mononuclear cells; **qPCR:** Quantitative reverse transcription polymerase chain reaction.

3.3.2. The link between an abnormal innate immune response and Ankylosing Spondylitis

One of the most intriguing aspects regarding AS pathogenesis is the possible link between pathogens and disease onset. There are several pieces of evidence that an abnormal host response against pathogens is implicated in AS and/or SpA pathogenesis. Sixty percent of patients with SpA without diagnosed Crohn's disease evidenced endoscopic or histological signs of gut inflammation [Mielants *et al.*, 1995]. Moreover, studies showing *HLA-B27* transgenic rats do not develop inflammatory intestinal or peripheral joint disease in a germ-free environment support a role of commensal gut flora in the shared pathogenesis of gut and joint manifestations [Taurog *et al.*, 1994].

Pattern recognition receptors (PRRs) in innate immune cells play a pivotal role in the first line of the host defense system. These receptors are transmembrane receptors such as Toll-like receptors (TLRs) or C-type lectin receptors (CLRs) and cytosolic receptors RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) [Jeong & Lee, 2011]. Interestingly, expression changes in genes involved in innate immune response such as *TLRs* [Assassi *et al.*, 2011], NLR family, pyrin domain containing 2 (*NLRP2)* [Sharma *et al.*, 2009] and C-type lectin domain family 4, member D *(CLEC4D)* [Pimentel-Santos *et al.*, 2011] were consistently observed in several different studies using microarray technology (Fig 3).

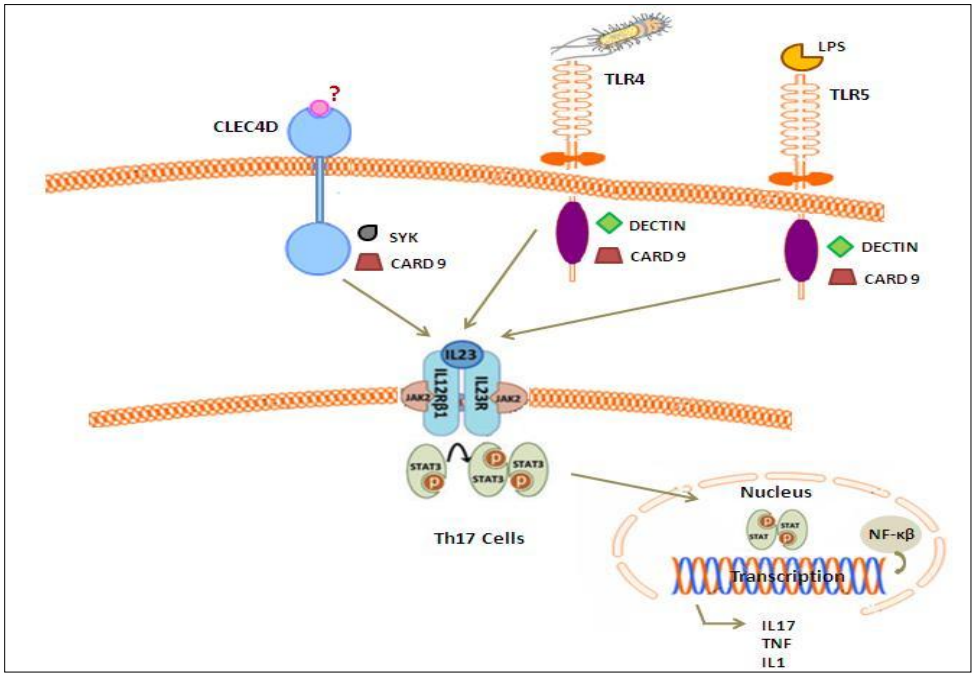

Figure 3: Possible functional effects between innate immune receptors and AS candidate genes (Adapted from Thomas & Brown, 2010a).

TLRs are characterized by an extracellular leucine-rich repeat (LRR) domain, a transmembrane domain and a cytoplasmic Toll/IL-1R (TIR) domain. As many as 13 TLRs family members have been identified in mammalian systems with TLRs 1 to 10 expressed in humans. They can be divided into 2 groups according to cellular localization and respective ligands. TLRs 1, 2, 4, 5, and 6, are expressed on the cell surface and recognize microbial components in the outer membrane of bacteria. TLRs 3, 7, 8 and 9 are found in intracellular vesicles and recognize microbial nucleic acids [Sirisinha, 2011]. TLRs are expressed in various immune (monocytes, macrophages, dendritic cells, B cells) and non-immune (epithelial cells, endothelial cells, fibroblasts) cells. *TLR4* was overexpressed in SpA patients in peripheral whole blood cells, assessed by microarray [Assassi *et al.*, 2011; Pimentel-Santos *et al.*, 2011], in PBMCs, measured by flow cytometry [de Rycke *et al.*, 2005] and in

lymphocytes, monocytes and neutrophils by qPCR [Yang *et al.*, 2007]. The main ligand for TLR4 is lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria however it also recognizes other exogenous pathogens such as mannan from *Candida albicans*, glycoinositolphospholipid from *Trypanosoma*, and the envelope proteins from mouse mammary tumor virus (MMTV) and respiratory syncytial virus (RSV). It also recognizes some endogenous molecules, including HSP (HSP60, HSP70, and HSP gp96), fibrinogen, oligosaccharides of hyaluronic acid, extracellular domain A of fibronectin, heparan sulfate, myeloid-related proteins (Mrp8 and Mrp14), oxidized LDL, saturated fatty acid and amyloid-β [Jeong & Lee, 2011]. Microarray analysis also showed overexpression of TLR5 in peripheral whole blood cells from SpA patients [Assassi *et al.*, 2011; Pimentel-Santos *et al.*, 2011]. Flagellin, a primary component of Gram negative bacteria flagella, is the main ligand for TLR5 [Hayashi *et al.*, 2001], which is mainly expressed on the luminar surface of epithelial cells in the mucosal tissues and respiratory tract [Gewirtz *et al.*, 2001].

The wide responsiveness of TLRs to a wide variety of external and internal signals, and the link that these receptors establish between the innate and adaptative immune systems, reinforces the theory that TLRs are strongly implicated in the development of chronic inflammatory diseases. However, mechanistic studies are needed in order to clarify the role of specific receptor subtypes in AS development.

Members of the NLRs family consist of a central nucleotide-binding and oligomerization (NACHT) domain, which is commonly flanked by C-terminal LRRs domain and N-terminal CARD or pyrin (PYD) domains [Schroder & Tschopp, 2010]. So far, 20 NLR family members have been identified in humans. Two main subgroups have been described. One, including NODs (NOD 1-5 and CIITA), detects pathogen-associated molecular patterns

(PAMPs) existing in Gram-negative bacteria cell walls and elicit responses that are distinct from those of the TLRs. The other NLR subgroup involves a large family of molecular complexes known as the "inflammasomes", the NLRPs (NLRP1-14) and the IPAF subfamily, consisting of IPAF and NAIP [Fitzgerald, 2010; Schroder & Tschopp, 2010]. The inflammasomes are macromolecular cytosolic complexes composed of several proteins, some of which are found in all inflammasomes (pro-caspase-1, Apoptosis-associated Speck-like Protein Containing a Caspase Recruitment Domain-ASC), and others which are present depending on the inflammasome type (cardinal, pro-caspase-5, domain with function to find-FIIND). These complexes are involved in the innate immune response recognizing both endogenous signals (adenosine triphosphate, urate, and calcium pyrophosphate crystals) as well as external pathogen-derived products (bacterial RNA, bacterial toxins) [Drenth & van der Meer, 2006].

As such, the reduced expression of *NLRP2* in AS was a very interesting observation [Sharma *et al.*, 2009]. *NLRP2*, as with other NLRs, induces an inhibition of the *NF-kB* gene, leading to regulation of IL1β, a relevant cytokine in the disease process. The downregulation of *NLRP2* may therefore lead to upregulation of *IL-1β*. Supporting this, polymorphisms in *NLR* genes have also been implicated in Behçet's disease and Crohn's disease which share some clinical features with AS [Kappen *et al.*, 2009; Cummings *et al.*, 2010]. Another interesting point is the association of *CARD9* with Crohn's disease and AS [Pointon *et al.*, 2010a] which has a pivotal role in NOD2 signaling.

Another family of receptors of particular interest is the C-type lectins which display a distinct protein domain, the carbohydrate recognition domain (CRD). Based on the organization of their CRDs, 17 distinct groups have been defined [Drickamer & Fadden, 2002; Zelensky &

Gready, 2005]. While some recognize DAMPs which facilitate adhesion between cells, adhesion of cells to extracellular matrix and other non-enzymatic functions, others may act as PRRs [Graham & Brown, 2009] after PAMP recognition. Upon ligand biding, CLRs can induce a variety of cellular responses, and can be functionally divided into those that inhibit or those that induce cellular activation. In general, inhibitory receptors contain a consensus immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domains, while activation receptors either contain an immunoreceptor tyrosine-based activation motif (ITAM), or associate with signaling adaptor molecules. Depending on whether signaling is through ITAM or ITIM , either activation of Src homology 2 (SH2) domain-containing protein tyrosine kinases (SyK, ZAP 10) or SH2 containing-phosphatases (SHP-1, SHP-2) are recruited, thereby up or downmodulating cellular activation, respectively [Long, 1999; Majeed *et al.*, 2001].

Genes encoding each family are distinctly clustered in the telomeric Natural Killer-gene complex (NKC), on chromosome 12. The Dectin-1 cluster of receptors includes *Dectin-1*, lectin-like oxidized low-density lipoprotein receptor-1 (*LOX-1*), C-type lectin-like receptor-1 (*CLEC-1*), *CLEC-2*, *CLEC12B*, *CLEC9A* and myeloid inhibitory C-type lectin-like receptor (*MICL*). The Dectin-2 cluster of receptors includes *Dectin-2*, *DCIR*, *DCAR*, *BDCA-2*, *Mincle* and *CLEC4D* [Graham & Brown, 2009].

Dectin-1, is expressed in dendritic cells, monocytes, macrophages, neutrophils and weakly in a subset of T cells, B cells and eosinophils. It recognizes fungal β-glucan, working as an activating receptor uniquely possessing an ITAM in the cytoplasmic domain. The induction of phagocytosis, production of reactive oxygen species and cytokine production is mediated by

NF-*kB* and spleen tyrosine kinase (Syk). In addition, some of these effects require cooperation with MyD88-mediated TLR signaling [Kanazawa, 2007].

Dectin-2 and *Mincle* are expressed in macrophages, dendritic cells and weakly in Langerhans cells and monocytes. The receptors recognize several pathogens (*Candida albicans, Saccharomyces cerevisiae, Mycoplasma tuberculosis, Histoplasma capsulatum*) but also endogenous ligands. Both have characteristic short cytoplasmic domains and are associated with FcRγ domains. Their activation, inducing the production of proinflammatory cytokines, is mediated by Syk- and CARD9-dependent pathways but independently of MyD88-mediated TLR signaling [Graham & Brown, 2009].

CLEC4D has been found to be expressed in a monocyte/macrophage restricted manner, and although no ligand or biological function has as yet been described, the receptor has been shown to be upregulated at the transcript level in a number of disease settings, similarly to two other members of the family, *Mincle* and *Dectin-2*. They are able to recognize and promote pathogen clearance and induce inflammatory signals. This process seems to follow the Syk and CARD9 pathway which was recently implicated in a mouse model of SpA [Ruutu *et al.*, 2010]. The upregulation of *CLEC4D*, observed for the first time in an expression profiling study of AS patients [Pimentel-Santos *et al.*, 2011], supports the importance of innate immune mechanisms in AS pathology. However, further studies are required to confirm this hypothesis.

3.3.3. Proinflammatory *vs.* immunosuppressive signatures

Transcriptional profiling studies have demonstrated that transcripts involved in the inflammatory response were differentially expressed in AS patients and controls, but reports on the nature of these changes seem to vary. A proinflammatory profile in PBMCs, from uSpA and AS, is indicated by an increased expression of regulator of G-protein signaling 1 *(RGS1),* Nuclear receptor subfamily 4, group A, member 2 *(NR4A2),* Heparin-binding EGF-like growth factor *(HBEGF)* and suppressor of cytokine signaling 3 *(SOCS3),* in both groups [Gu *et al.*, 2009]. However, other reports suggest decreased immune responsiveness such as a "reverse Interferon-gamma (IFNγ) signature" [Smith *et al.*, 2008], and immunosuppressive phenotypes [Duan *et al.*, 2010; Pimentel-Santos *et al.*, 2011]. The main reason for these differences in the transcriptomic profiles, between the first study and the 3 later studies, is unknown but differences in patients and methodologies may contribute.

IFNγ dysregulation in AS is supported by previous studies of cytokines expression. A lower frequency of IFNγ positive T cells has been reported in AS patients [Rudwaleit *et al.*, 2001] and gut biopsy samples show a reduced TH1 profile in lymphocytes from SpA patients [van Damme *et al.*, 2001]. Moreover, IFNγ is expressed at lower levels in synovium from SpA compared to rheumatoid arthritis patients [Canete *et al.*, 2000]. This knowledge may contribute to understanding AS pathogenesis as decreased IFNγ production by macrophages could impair the host's ability to clear pathogenic organisms. Recent studies support this theory [Rothfuchs *et al.*, 2001; Inman *et al.*, 2006], and may implicate arthritogenic organisms in AS susceptibility. In addition, IFNγ reduction, can contribute to activation of the IL-23/IL-17 axis a major axis in AS pathogenesis.

Complementary to the report in macrophages from peripheral blood of AS patients [Smith *et al.*, 2008], two different studies, from PBMCs and whole blood, have shown an immunosuppressive phenotype [Duan *et al.*, 2010, Pimentel-Santos *et al.*, 2011]. The first one validated three downregulated genes, *NR4A2*, Tumor necrosis factor, alpha-induced protein 3 *(TNFAIP3)* and CD69 molecule *(CD69)*. *NR4A2* has been associated with T-cell subset communication and the macrophage inflammatory response. TNFAIP3 serves as negative feedback system for the TNFα induced by NF-*kB*, acting as an anti-inflammatory molecule to control prolonged inflammation. CD69 is an early leukocyte activation molecule expressed at sites of active inflammation. Of further interest were the results of Ingenuity Pathways Analysis using the differentially expressed gene set showing altered activity of the JAK/STAT signaling pathway in AS patients [Duan *et al.*, 2010]. Both *STAT3* and *JAK2* have been shown to be genetically associated with IBD and AS [Barrett *et al.*, 2008; Danoy *et al.*, 2010; Evans *et al.*, 2011], and represent key downstream molecules of the IL-23/IL-17 pathway [Ma *et al.*, 2008].

In the second study downregulation of several pro-inflammatory genes were described highlighting another aspect of AS pathogenesis [Pimentel-Santos *et al.*, 2011]. Protein tyrosine phosphatase, non-receptor type 1 *(PTPN1)* and Dedicator of cytokinesis 10 *(DOCK10)*, which are both involved in mediating IL4 actions [Paul & Ohara, 1987] were downregulated. Protein tyrosine phosphatase 1B (PTP1B), the *PTPN1* protein product, is a ubiquitously expressed enzyme shown to negatively regulate multiple tyrosine phosphorylation-dependent signaling pathways, including the downstream processes involved in CLRs activation [Long, 1999; Majeed *et al.*, 2001] and IL4 signaling [Lu *et al.*, 2008]. Dock10 is also regulated by IL4 in B cells [Yelo *et al.*, 2008]. This is of particular interest as

IL4 may play a role in AS pathogenesis. Interleukin 4 (IL4) has a variety of stimulatory and inhibitory actions on B and T cells [Jelinek & Lipsky 1988; O'Garra *et al.*, 1988; Rousset *et al.*, 1988]. Recent studies have also indicated a potential role for IL4 producing CD8+ T cells in the pathogenesis of AS. Although CD8+ T cells are predominately associated with the production of 'TH1' cytokines, such as IFN*γ*, there is now good evidence that some subsets of these cells can also produce 'TH2' cytokines such as IL4, IL5 and IL10 [Baek *et al.*, 2008]. The potential functions associated with IL4-producing CD8+ T cells are as yet unclear but the subtype CD8+/TCR αβ+ T cells, with a regulatory phenotype and function (expressing CD25+, CTLA4+, Foxp3+, but negative for IFNγ and perforin), were previously described in peripheral blood of AS patients [Jarvis *et al.*, 2005]. These results were confirmed in a recent study suggesting an altered pattern of CD8+ T cell differentiation in AS and in *HLA-B27*+ healthy individuals. This predisposition to generate IL4+CD8+ T cells may play a role in pathogenesis of SpA [Zhang *et al.*, 2009]. Further supporting this theory, *RUNX3* was identified as a candidate gene in a GWAS [TASC, 2010]. The association of *RUNX3* with AS provides additional evidence of a role for CD8+ T cells in the disease. Its expression in immature lymphocytes is triggered by IL7R signaling, leading to suppression of CD4 and upregulation of CD8 expression [Park *et al.*, 2010].

Although there are some differences between the different expression profiling studies, their findings do contribute to a greater understanding of the pathogenesis of AS, particularly in the delineation of the roles of the innate and adaptive immune responses.

3.3.4. Bone ossification and resorption processes

Bone formation and bone loss take place at sites closely located to each other presenting an "apparent paradox", which is reflected in the changes in bone and cartilage metabolism occurring in the AS disease process [Carter & Lories, 2011]. Ossification is the hallmark of AS and has been linked to aberrant activation of bone morphogenic protein (BMP) and wingless (WNT) signaling. Bone resorption, driven by the impact of inflammation on the bone remodeling cycle, occurs simultaneously, with up to 56% of patients becoming osteopenic and some of them osteoporotic [Lange *et al.*, 2005].

Biomarkers, reflecting structural damage and disease activity, constitute a high priority for the understanding of the pathogenesis of AS and for the new therapy discovery. Two microarray-based studies have contributed to the improvement of knowledge in this field. A bone remodeling signature was described associated with an overexpression of *BMP6*, Proprotein convertase subtilisin/kexin type 6 *(PCSK6)*, Kringle containing transmembrane protein 1 *(KREMEN1)* and Catenin (cadherin-associated protein) alpha-like 1 *(CTNNAL1)* genes in SpA patients [Sharma *et al.*, 2009].

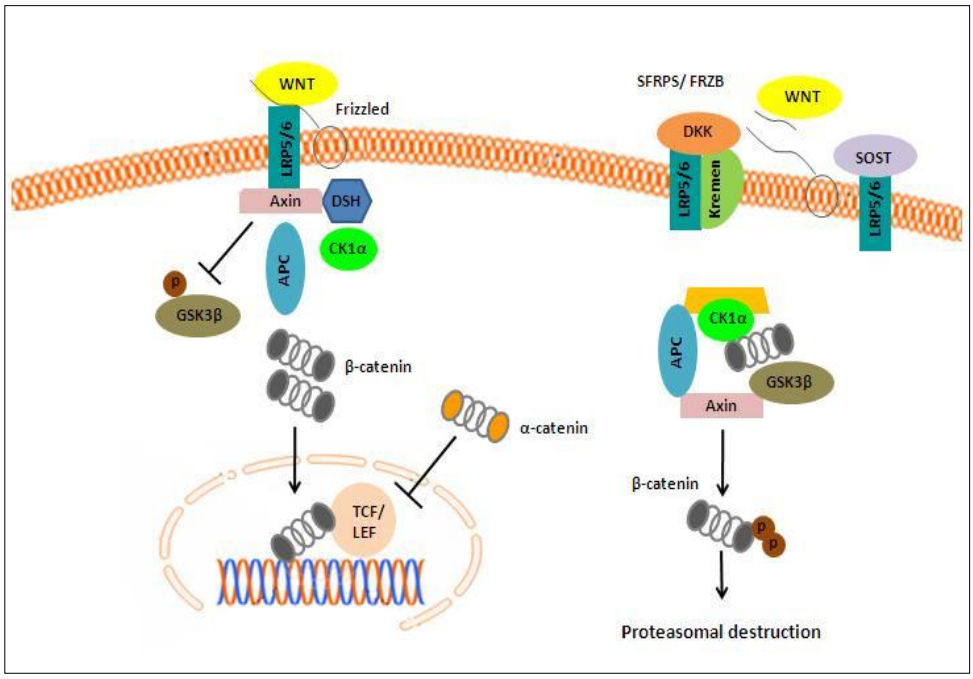

Figure 4: The canonical *WNT* signaling pathway (adapted from Carter & Lories, 2011).

KREMEN1 and *CTNNAL1* are negative regulators of WNT/catenin pathway via dickkopf homolog 1 (DKK1), or by direct inhibition of β-catenin, respectively (Fig 4). Although four different intracellular pathways can be triggered upon WNT receptor interaction, the WNT/β-catenin or "canonical" pathway is of particular interest in bone and cartilage biology. This pathway involves the interaction of WNT ligands with frizzled (FZD) receptors and low-density lipoprotein receptor-related protein 4, 5 or 6 (LRP 4, 5 or 6) co-receptors. In the absence of a WNT-FZD-LRP 4/5/6 interaction, cytoplasmic β-catenin is captured within a destruction complex comprising adenomatous polyposis coli (APC), axin, glycogen synthase kinase 3β (GSK-3β), and casein kinase 1α (CK1 α). The kinases phosphorylate β-catenin, which leads to ubiquitinylation and subsequent destruction in a proteasome complex. When WNT does complex with FZD and LRP 4/5/6, axin binds to the cytoplasmic tail of LRP5 or 6, thereby phosphorylating and inhibiting GSK-3β [Gordon & Nusse, 2006]. This process

enables cytoplasmic β-catenin accumulation which then translocates to the nucleus, where it interacts with transcription factor (TCF)/lymphoid enhancer factor (LEF) family members and modulates WNT target genes expression [Gordon & Nusse, 2006]. Several proteins that are not involved in β-catenin stability can also regulate β-catenin signaling. One example is the direct association of α-catenin with β-catenin in the nucleus which interferes with protein-DNA interactions required for TCF-mediated transcription [Giannini *et al.*, 2000]. In addition, different endogenous antagonists inhibit WNT signaling; DKK1 and sclerostin (SOST). DKK1 acts by direct binding to and inhibiting the WNT co-receptor LRP6. The related DKK2, however, can function either as LRP6 agonist or antagonist, depending on the cellular context, suggesting that its activity is modulated by unknown co-factors. In this context, the transmembrane proteins KREMEN1 and -2 were recently identified as additional DKK receptors, which bind to both DKK1 and DKK2 with high affinity [Mao & Niehrs, 2003]. It was shown that DKK1 was able to simultaneously bind to LRP5/6 and KREMEN and that the ternary complex was rapidly endocytosed, thus preventing the WNT-LRP interaction. The interaction with KREMEN seems not to be essential, but it plays a role in facilitating DKK-mediated antagonism if the level of LRP5/6 is high [Wang *et al.*, 2008]. The upregulation of *KREMEN1* and *CTNNAL1* genes by these mechanisms can compromise bone formation. In contrast, upregulation of *BMP6* and its regulator *PCSK6* can contribute to the AS ossification process. BMPs are members of the transforming growth factor- β (TGF β) superfamily, play a crucial role in embryonic development, cell lineage determination, and osteoblastic differentiation and function. Enthesitis, a distinctive feature of SpA, is associated with heterotopic cartilage and bone formation (enthesophyte) [Benjamin & McGonagle, 2001]. Different BMPs are expressed in distinct stages of ankylosing enthesitis shown in the DBA/1 mouse model. BMP2 is found in proliferating cells and entheseal cells committing their

differentiation fate to chondrogenesis. BMP7 is recognized in prehypertrophic chondrocytes and BMP6 in hypertrophic chondrocytes [Lories *et al.*, 2005]. Several regulators of endochondral bone formation with different effects in different stages were described [Kronenberg, 2003]. It is therefore possible that the presence of progenitor cells at the entheseal site promotes bone formation in SpA patients. Activation of the BMP signaling pathway (phosphorylated Smad1/5) was found in cells at the sites of entheseal inflammation in patients with AS [Lories *et al.*, 2005].

Another bone remodeling signature was identified in association with a downregulation of Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2 *(SPOCK2)*, E1A binding protein p300 *(EP300)* and protein phosphatase 2, regulatory subunit A, alpha *(PPP2R1A)* in AS, which are possible mediators in the ossification process [Pimentel-Santos *et al.*, 2011].

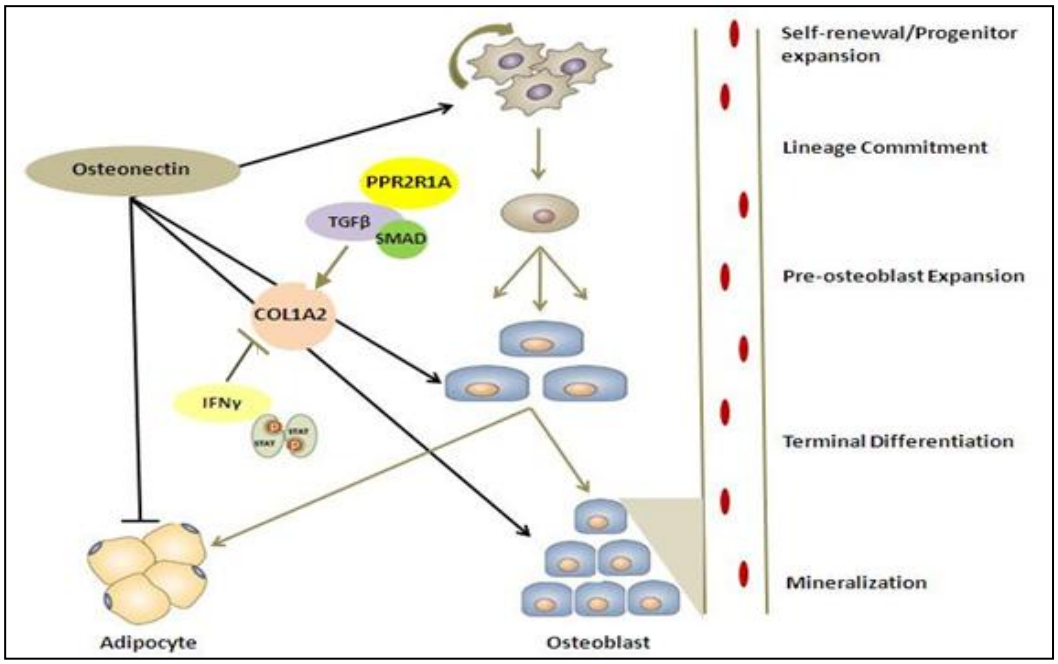

Figure 5: Model representing the effects of *SPARC* on marrow mesenchymal progenitors (adapted from Delany & Hankenson, 2009).

SPOCK2, also known as Sparc/osteonectin, is a non-collagenous bone protein. It is a member of the matricellular class of glycoproteins which includes periostin, tenascin C, osteopontin, bone sialoprotein, thrombospondin-1 and thrombospondin-2 [Alford & Hankenson, 2006]. It has been hypothesized to play a role in the regulation, production, assembly and maintenance of the matrix turnover in cartilage [Hausser *et al.*, 2004; Gruber *et al.*, 2005]. In this process TGFβ and IFNγ exert antagonistic effects, and play important role in the physiologic regulation of extracellular matrix turnover. In fact, the *TGFβ* gene positively regulates pro-alpha2 chain of type I collagen (COL1A2) through the Smad signal transduction pathway, whereas *IFNγ* inhibits COL1A2 through Stat1. Additionally, *PPP2R1A* also downregulated in AS [Pimentel-Santos *et al.*, 2011], is thought to mediate TGF*β* regulation through Smad [Heikkinen *et al.*, 2010]. Animal models using *SPARC*-null mice have provided excellent information on the function of this protein in bone. *SPARC*-null mice develop profound low-turnover osteopenia (bone loss), associated with decreased numbers of osteoblasts and osteoclasts, and a markedly decreased bone-formation rate [Delany *et al.*, 2000; Boskey, 2003]. Moreover *SPARC*-null mice have decreased trabecular bone volume due to decreased trabecular number [Machado dos Reis *et al.*, 2008] and an increase in extra-skeletal adipose deposits [Mansergh *et al.*, 2007]. *In vitro* studies showed accumulation of SPARC during early osteoblastic differentiation, likely in association with collagen matrix, which decreases as the cells acquire more osteoblastic characteristics. This expression pattern seems appropriate because SPARC regulates collagen fibril assembly, and matrix is abundantly deposited in the earlier stages of differentiating cultures. *SPARC* has a positive effect on maintaining and expanding the mesenchymal progenitor pool, and promotes osteoblastogenesis/osteoblast function and decreases adipogenesis (Fig 5) [Delany & Hankenson, 2009]. Expression of *SPARC* by osteoclasts has not been reported. Therefore, the

mechanisms by which *SPARC* limits osteoclast formation may involve the direct interaction with osteoclasts or osteoclast precursors through the bone matrix, and/or the effect of *SPARC* on immune cells, marrow stromal cells, and osteoblasts supporting osteoclast development [Machado do Reis *et al.* 2008]. In summary, recent findings supports the idea that *SPARC* play a critical role in regulating bone remodeling and maintaining bone mass. Thus its dysregulated expression may contribute to the aberrant matrix formation in AS.

Interestingly, the protein produced by *EP300* belongs to the group of nuclear p300/CBP transcriptional coactivators for both Smad3 and Stat1a that integrate signals that positively or negatively regulate *COL1A2* transcription [Ghosh *et al.*, 2001]. Transactivated p300, controlled by phosphoinositide-3 kinase (PI3K)/AKT, is also an important transcriptional co-activator of Sox9, which modulates the expression of the major extracellular matrix component, aggrecan [Cheng *et al.*, 2009]. Moreover, there is some evidence supporting a p300 interaction with the Wnt pathway as it is a β-catenin transcriptional coactivator. Downregulation of these genes might lead to a loss of matrix integrity thereby accelerating tissue damage. This may be reinforced by a pro-inflammatory status associated with downregulation of *EP300* [Ahmad *et al.*, 2007].

3.3.5. Biomarkers for early diagnostic purposes

Low back pain (LBP) is a very common symptom, responsible for 3% of annual medical visits in the USA [Licciardone, 2008]. However only 5% of the chronic back pain seen in general practice designated as "inflammatory", is associated with SpA [Underwood & Dawes, 1995]. To classify patients with AS or SpA, various criteria sets can be used. The modified New York Criteria [van der Linden *et al.*, 1984a] for AS, the Amor criteria [Amor *et al.*, 1990] and the European Spondyloarthropathy Study Group (ESSG) criteria [Amor *et al.*, 1991], developed in the 1990s, before magnetic resonance imaging (MRI) was available, addressed all SpA subtypes. Recently, it has been proposed to divide SpA patients into subgroups according to clinical presentation. The Assessment of SpondyloArthritis International Society (ASAS) group has developed criteria to classify patients with axial SpA with or without radiographic sacroiliitis, and patients with predominant peripheral SpA [Rudwaleit *et al.*, 2009b, 2009c; Rudwaleit, 2010a, 2010b]. With a sensitivity of 82.9% and a specificity of 84.4% , these axial SpA criteria perform better than the ESSG and Amor criteria, even after adding "sacroiliitis on MRI" to the latter. The peripheral criteria with sensitivity of 77.8% and specificity of 82.8% are also promising for use in clinical practice [Rudwaleit, 2010a, 2010b]. The ASAS criteria have been developed as classification criteria but they are likely be useful as diagnostic criteria, especially in patients with non-radiographic axial SpA at an outpatient rheumatology clinic [van den Berg & van der Heijde, 2010]. This may help to make an early diagnosis and prevent the current diagnostic delay, described as 5 to 10 years between the first occurrence of symptoms and an AS diagnosis [Feldtkeller *et al.*, 2003; Haibel *et al.*, 2007]. It prevents unnecessary diagnostic tests and more importantly

makes it possible to commence more effective therapies earlier. This is crucial as at early disease stages, even those without definite radiologic sacroiliitis, can suffer as much pain and have as high a disease activity as patients with established AS [Rudwaleit *et al.*, 2009a]. Therefore, it's important to consider all patients with SpA with predominantly axial involvement irrespective of the presence or absence of radiographic changes as belonging to one disease continuum [Rudwaleit, 2005]. Despite all these advantages with the new ASAS criteria, one of the major reasons for diagnosis delay is a low awareness of AS among physicians in primary care [Sieper, 2009]. In this particular setting, several concerns have been raised regarding the use of ASAS criteria for diagnostic purposes [van den Berg & van der Heijde, 2010]. Thus current diagnosis of AS and SpA still relies on clinical and imaging parameters that may be relatively complex for general use in primary care. Screening parameters for an early referral of AS patients, easy to apply by the non-specialist, sensitive, specific and not too expensive, should be identified. For the rheumatology community this represents a great challenge. Expression studies can identify a small number of genes whose expression profile might serve as cost effective set of surrogate biomarkers for AS.

One study has identified a small number of genes whose expression profile might serve as a cost-effective set of surrogate biomarkers for AS and uSpA [Gu *et al.*, 2009]. In this PBMC-based microarray study, all included patients fulfilled Calin criteria for inflammatory back pain and were taking NSAIDs and/or SSZ. They concluded that the overall gene expression was higher in uSpA than in AS patients and identified a member of the family of *RGS1* as the most promising biomarker for uSpA and AS, with this gene more highly expressed in SpA than in AS. They demonstrated a receiver operating characteristic (ROC) area under the curve (AUC) range between 0.93-0.99. Biomarkers with ROC AUC 0.8-1.0 are usually considered

to be useful in clinical practice [Rao, 2003]. To evaluate arthritis related factors that might enhance RGS1 expression, a panel of 25 cytokines and chemokines on a monocyte derived human cell line were used. The 2 strongest activators of RGS1 expression were TNFα and IL-17. However, in order to be implemented in clinical practice further studies are clearly required. It requires a multicenter, multi-ethnic validation but also comparison with results obtained through MRI and the new ASAS classification criteria. There are several other concerns. This gene was differentially expressed between AS patients and healthy controls, in another microarray study PBMC based [Duan *et al.*, 2010], but contrary to the first study it was underexpressed. Finally, it wasn't identified as differentially expressed in a recent published study from a well defined population of Portuguese ethnicity background [Pimentel-Santos *et al.*, 2011]. These distinct results reinforce the need for larger studies involving different ethnic groups.

3.3.6. Gene expression changes after anti-TNFα therapy

Biomarkers that allow quantitative assessment of treatment response have great potential in clinical practice. They enable appropriate choice of therapy, drug dosage to maximize effect and minimize toxicity, and monitor disease outcomes representing the foundation of evidence-based medicine [de Vlam, 2010]. The introduction of biologic therapies targeting TNFα (infliximab, etanercept, adalimumab, golimumab) has changed clinical practice with several benefits regarding clinical management and prognosis. Additionally, the scientific community is waiting for the market introduction of new biological treatments with new targets in the near future. Identification of markers of treatment response would be of great clinical benefit by facilitating better targeting of these treatments to those most likely to respond, and potentially significantly reduce treatment costs by minimizing use of these expensive agents in patients unlikely to respond.

Until now the Visual Analogue Scale (VAS) pain, VAS general health, BASDAI, inflammatory parameters and composite response criteria are used to evaluate treatment effect in AS. ASAS defined and validated three levels of response: ASAS20, ASAS40, and ASAS partial remission, for patients treated with non-steroidal anti-inflammatory drugs and TNFα blockade [Anderson *et al.*, 2001]. The recent introduction of the ASDAS criteria [van der Heijde *et al.*, 2009] seems to be a highly discriminatory instrument for assessing AS disease activity and monitoring changes in disease and is finding good use in clinical practice. However all these criteria aren't predictors of response to therapy and rely on subjective self-evaluation and are not free from disease-unrelated influences, so biomarkers with high sensitivity and specificity for treatment response are highly desirable.

Current markers of response such shorter disease duration, HLA-B27 carriage, elevation of acute phase reactants (CRP), and marked spinal inflammation, as shown by MRI, may be predictors of good response; conversely, longer disease duration, structural damage and poor function may be predictors of poor- or non-response [Rudwaleit *et al.*, 2004, 2008]. Data from the British Society of Rheumatology Biologics Register has shown raised inflammatory markers at the start of therapy predicted a greater improvement in disease activity [Lord *et al.*, 2010]. Predictors of improvement in function, measured using the BASFI, have shown a strong association with gender (significantly greater improvement in women) and concurrent disease-modifying antirheumatic drugs (DMARDs) therapy [Lord *et al*, 2010]. Finally, prevention of damage is another important outcome of therapy. Slow radiographic progression of the disease and the relatively small fraction of patients progressing over a period of 2-3 years make radiographic evaluation less sensitive for damage evaluation. However, the major predictor of progression is previous existing radiographic damage. While it is clear that anti-TNFα agents have a structural benefit in inflammation-mediated resorptive damage as indicated by changes in bone and cartilage metabolism, an effect on radiographic progression remains to be demonstrated in AS [de Vlam, 2010]. A study of the relationship of biomarker levels, disease activity and the spinal inflammation detected by MRI was performed in patients with AS receiving Infliximab over a 24 week period. Early reductions in IL-6 (by week 2) but not CRP or Vascular endothelial growth factor (VEGF), were significantly associated with reductions in MRI activity and BASDAI scores by week 24 in the infliximab group [Visvanathan *et al.*, 2008]. However the structural changes of this effect are not known.

Gene expression profiling constitutes a widely available and promising technology to identify treatment-associated changes. In two recent studies it was demonstrated that anti-TNF α treatment leads to significant alteration of gene expression and protein profiles, supporting the use of systematic gene expression and proteomic analysis to shed new light on pathogenic pathways with importance in the chronic inflammation of AS [Haroon *et al.*, 2010; Grcevic *et al.*, 2010]. Anti-TNFα therapy induced a rapid change in the expression profile within 2 weeks in AS patients with down-regulation of lymphotoxins exhibiting inducible expression and competing with herpes simplex virus glycoprotein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes *(LIGHT*)**,** interferon α receptor 1 ***(****IFNAR1)*, interleukin 17 receptor *(IL17R)* and erythropoietin receptor *(EPOR)* genes. *LIGHT*, a member of the TNF superfamily, was the most significantly down-regulated gene and serum soluble LIGHT levels correlate well with other inflammatory markers such as, CRP and ESR. However, no significant differences between responders and non-responders were observed in either *LIGHT* mRNA expression or LIGHT serum levels. A time gap between changes in inflammatory mediators and improvements in subjective disease severity scoring metrics may explain these findings [Haroon *et al.*, 2010]. Although these results are interesting more studies are needed for validation. Another study using peripheral blood expression profiles based on PBMCs cells assessed several bone-regulatory factors as potential discriminators of different forms of arthritis, disease activity and therapy responsiveness [Grcevic *et al.*, 2010]. ROC curve analysis suggested higher expression of Runx2 was a potential molecular maker for AS. Although no increased gene expression of *BMP4* or *LIGHT* in AS patients compared with healthy controls were seen, higher expression was evident in AS patients resistant to conventional therapy. Thus *LIGHT* might be considered an interesting biomarker to consider in future studies.

Another marker which must be considered for a treatment-response marker is the CX3CL1-CC3CR1 complex. In RA, CX3CL1 levels decline in patients showing a clinical response to infliximab treatment. Moreover, patients with active RA who did not show a clinical response to infliximab showed higher basal CX3CL1 levels than those who did [Odai *et al.*, 2009]. These results suggest that the CX3CL1-CX3CR1 in patients with active RA may be sensitive to anti-TNFα therapy and confirm that CX3CL1 plays a crucial role in the pathogenesis of RA, although further investigations are required. These results suggest that CX3CL1-CX3CR1 may be also relevant in AS process. This is further supported with the underexpression of this gene in AS patients [Pimentel-Santos *et al.*, 2011].

4. FINAL REMARKS

The present research work made it possible to characterize AS in Portugal. The key-findings were: higher prevalence in males than in females (1.6:1); eight years delay from onset of symptoms and diagnosis; predominant axial involvement; AAU as the main extra-articular manifestation; high activity and severity of disease (BASDAI, BASFI and BASMI scores >4); widespread use of NSAIDs; low prescription of anti-TNFα agents; and low/moderate positivity for *HLA-B27*.

The validation of Bath indices (BASDAI, BASFI, BASMI and BASG) in Portuguese version, was relevant for proper monitoring of patients. These indices were widespread and are currently being used as a standard.

The newly defined BASDAI, BASFI, BASMI, and mSASSS charts for Portuguese patients allow for both comparisons of Portuguese patients to other ethnic groups and to monitor national patients over different periods of time. In a near future, proper validation of these charts will also allow to use them on an individual basis, meaning that patients may be monitored conviniently in clinical practice in a supplementary way.

Genetic studies performed in Portugal validated genes that have also been previously identified in others ethnic groups; despite the relevance of MHC on AS susceptibility, primarily related to the *HLA*-B27, others non-MHC genes have been implied, such as *ERAP1* and *IL23R*. Furthermore, new genes with interest in AS susceptibility and in new therapeutical targetting were also documented. The identification of one *TNFSF8* polymorphism in French population, then validated in the Portuguese and Belgian

populations, constitutes a new potential interesting finding to be confirmed in future studies. This research identified two *HLA-B27* haplotypes, *A*02/B*27/Cw*02/DRB1*01/DQB1*05* confering susceptibility to AS, and *A*02/B*27/Cw*01/DRB1*08/DQB1*04* providing protection in terms of disease activity, functional and radiological repercussion. Finally, validation of gene expression signature of AS from whole blood opens new and exciting perspectives into selecting highly discriminant biomarkers.

From a research point of view, the present genetic identification also pointed out sound pathophysiologic pathways for disease initiation and progression.

Adding the most relevant genetic markers to clinical and imagiologic parameters may yield diagnostic methods not only much more robust and specific but also more adapted to each patient – the final challenge in the quest of personalized medicine.

5. FUTURE PERSPECTIVES

A recent and huge technology progress in the present field of research has been encompassed with great enthusiasm for the numerous discoveries achieved.

There is now convincing evidence that AS assumes different patterns, clinically and genetically, according to ethnic groups considered. It was therefore mandatory to obtain data from Portuguese population.

Future work in Portuguese context should be two-folded and aim at:

a) Collecting new data from patients involved in the 2007 study; creating a cohort will enable to conduct prospective studies;

b) Enlarging the database by enrolling further rheumatologic centres as to ascertain a higher results' reliability. This last future goal is vital to obtain sound conclusions, to compare different ethnicities worldwide, and ultimately participate in international study consortia.

During the current study, new research goals and questions have arisen. It is important to validate the charts for BASDAI, BASFI, BASMI, and mSASSS and assess their applicability on an individual basis. The analysis of AS economic burden in national population is yet another relevant aspect to complete. How does time influence the disease? It will be undoubtly interesting to compare current data with future information harvested after a five-year period (2007-2012). In addition, next future work includes:

a) Population stratification into different subgroups according to relevant phenotypic characteristics;

b) Sellect the most significant gene variants associated with AS susceptibility, proceed with functional analysis (animal models and/or cellular lineage experimenting) and study the influence of specific genes on phenotypic characteristics;

c) Promote studies to screen patients and controls with different methodologies - RNA microarray, proteomic, and epigenetic studies - and thereafter try to integrate results. This could be an interesting means of identifying the most relevant "players" in pathophysiologic pathways of disease.

As an ultimate goal, one might expect to establish an algorithm with clinical, imaging, and genetic data input to determine disease presence at an early stage, implement treatment as soon as possible and, therefore, improve long term prognosis.

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“Give me a place to stand, and I will move the Earth.”

Archimedes

7. ANNEXES

ANNEXE I - RESUMO ALARGADO EM PORTUGUÊS

1. INTRODUÇÃO

A Espondilite Anquilosante (EA) é a doença de referência dentro do grupo das espondilartrites seronegativas (SpA), no qual se incluem também as artrites associadas a doenças intestinais inflamatórias (IBD), a artrite psoriásica, as artrites associadas a uveíte anterior aguda (AAU), as artrites reativas e as espondilartrites indiferenciadas (uSpA) [Rudwaleit *et al.*, 2009a; 2010a; 2010b]. Estas doenças partilham diversas características como o envolvimento articular (axial e/ou periférico), o envolvimento extra-articular (entesopático e específico de órgão), a seronegatividade para o fator reumatoide e a forte associação ao HLA-B27 [Dougados *et al.*, 1991; Calin & Taurog, 1998]. Neste contexto, a dor lombar inflamatória representa o "sintoma chave", a neo-formação óssea, traduzida pelo aparecimento de sindesmofitos conducentes à anquilose, a "imagem de marca" e a inflamação das *entesis* a "alteração histopatológica" característica da doença [Benjamin & McGonagle, 2001].

A sua incidência e prevalência tendem a espelhar a prevalência do HLA-B27 [Khan, 1996]. Entre adultos positivos para a presença de HLA-B27, a prevalência de SpA foi estimada em 4.5% e a de EA em 1.6%, [Benevolenskaya *et al.*, 1996]. Nos Caucasianos, onde a positividade para o HLA-B27 é de 8%, a prevalência das SpA é de 1.3% e da EA oscila entre 0.2-0.9% [Braun *et al.*, 1998]. Em Portugal, estimou-se uma prevalência de 0.6% para a EA

[ONDoR, 2003-2005]. Assim, as SpA como um todo, e a EA em particular, encontram-se entre as formas mais frequentes de artrite.

A incidência da EA tem também sido estudada em várias populações, registando-se algumas discrepâncias. Valores similares de 6,4; 6,9; 7.26; 7.3 por 100,000 pessoas/ano foram estimados nas populações da República Checa [Hanova *et al.*, 2010], da Finlândia [Kaipiainen-Seppanen *et al.*, 1997], da Noruega [Bakland *et al.*, 2005] e de Rochester-EUA [Carbone *et al.*, 1992], respetivamente. Na população da Grécia porém, a incidência parece ser significativamente mais baixa (1,5 por 100,000) [Alamanos *et al.*, 2004]. Apesar da dificuldade em se avaliar de forma exacta a incidência e a prevalência da EA, devido à heterogeneidade étnica das populações, à dificuldade na aplicação dos critérios de diagnóstico e à natureza transitória de alguns sintomas [Reveille, 2011], parece haver em termos globais, uma relativa estabilidade destes parâmetros, pelo menos nos anos mais recentes [Gabriel & Michaud, 2009].

Em termos clínicos, a EA afeta tipicamente indivíduos jovens, iniciando-se os primeiros sintomas antes dos 30 anos em 80% e após os 45anos, em menos de 5% dos doentes [Feldtkeller *et al.*, 2003]. Os homens tendem a ser mais frequentemente afetados que as mulheres, sendo no entanto, as proporções descritas variáveis oscilando de 2:1 [Feldtkeller *et al.*, 2003, Brunner *et al.*, 2002], 3:1 [Lee *et al.*, 2007] até 6-8:1, como descritas em algumas populações Asiáticas [Lee *et al.*, 2002; Zeng *et al.*, 2003; Jung *et al.*, 2010]. O mecanismo exacto subjacente a estas diferenças não é conhecido, mas o fator género parece exercer influência nas manifestações clínicas e no próprio prognóstico da doença.

No espetro dos sintomas incluem-se a dor lombar inflamatória e/ou a oligoartrite periférica assimétrica (com envolvimento predominante dos membros inferiores), a entesite e o

envolvimento específico de órgão. A AAU, a psoríase e a doença inflamatória crónica do intestino são manifestações frequentes contrariamente ao envolvimento pulmonar, renal, neurológico e cardíaco [Braun & Sieper, 2007]. É importante, no entanto, referir que de acordo com vários estudos realizados na Europa e na Ásia, os doentes com EA têm maior prevalência de co-morbilidades comparativamente com a população saudável [Bremander *et al.*, 2011]. Em Taiwan, foi documentado um aumento do risco de doença cardiovascular, neurológica, pulmonar, gastrointestinal, endócrina, hematológica e mental. A hipertensão (16.4%), a úlcera péptica (13.9%) e as cefaleias (10.2%) assumem-se como as complicações mais prevalentes [Kang *et al.*, 2010]. O risco aumentado para doença cardiovascular tem sido um achado consistente, estando provavelmente relacionado com o processo de inflamação crónica e com a elevada prevalência de fatores risco cardiovasculares nestes doentes [Boonen *et al.*, 2002; Bakland *et al.*, 2011; Mathieu *et al.*, 2010].

A EA associa-se ainda a um aumento da mortalidade de 50% comparativamente com indivíduos saudáveis, do mesmo sexo e idade [Boonen *et al.*, 2002]. Um estudo recente, baseado na avaliação de índices de mortalidade estandardizada, mostrou porém que o risco só se encontra significativamente aumentado entre os doentes do sexo masculino [Bakland *et al.*, 2011]. Estes apresentam também mais alterações estruturais (incluindo a coluna de bamboo), valores mais baixos de densidade mineral óssea [Karberg *et al.*, 2005] e maior número de fraturas [Cooper *et al.*, 1994], o que pode contribuir para uma cifose mais grave [Vosse *et al.*, 2006], comparativamente com os doentes do sexo feminino. Torna-se assim evidente que é importante avaliar de forma sistemática, o envolvimento articular e sistémico em cada indivíduo, para pôr em evidência o real impacto da doença, para o doente e para a sociedade.

Neste contexto, compreende-se facilmente que a EA possa levar a uma deterioração da função física e da qualidade de vida (QoL), condicionando um aumento da incapacidade para o trabalho e paralelamente, do consumo de serviços de saúde. Para se pôr em evidência o impacto da doença, efetuaram-se comparações entre doentes e a população geral [Ware, 2000], usando medidas genéricas de QoL, como o Medical Outcomes Study Short Form-36 (SF-36) [Guyatt *et al.,* 1993]. Em diversos estudos efectuados, confirmou-se que a EA condiciona uma redução significativa da QoL comparativamente à população geral, o que se deve essencialmente ao compromisso dos domínios físicos (e em menor escala dos domínios da esfera psicossocial) [Dagfinrud *et al.,* 2004; Davis *et al*., 2005; Singh & Strand, 2009]. Isto significa que o componente físico será o principal determinante da morbilidade associada à EA. Como muitos dos fatores implicados - gravidade e duração da doença, resposta à terapêutica e efeitos adversos, co-morbilidades, fatores socioeconómicos e acesso aos serviços de saúde - são fatores modificáveis, é de supor que intervenções específicas possam melhorar a função e a QoL destes doentes [Singh & Strand, 2009].

Foram descritos elevados níveis de incapacidade para o trabalho com grande impacto na produtividade e nos custos sociais da doença [Boonen *et al.,* 2002]. Na Holanda, dentro do grupo de doentes com EA, a percentagem de trabalhadores é 11% mais baixa e os níveis de incapacidade para o trabalho 15% mais elevados que na população em geral. No subgrupo de doentes com trabalho remunerado o número médio de dias de baixa relacionados com a EA foi de 10 dias/ano por doente [Boonen *et al.,* 2001].

Em termos de utilização de serviços de saúde, os resultados de alguns estudos Europeus, apontam para um consumo entre 1,4-4,0 consultas gerais e 1,7-2,8 consultas de especialidade/ano devido a problemas relacionados com a EA [Boonen et al, 2003a, 2003b,

2005; Verstappen et al., 2007]. Resultados semelhantes foram obtidos nos EUA com valores superiores a 2,1, para consultas gerais e de especialidade/ano [Ward, 2002]. Outros estudos, ao utilizarem bases de dados administrativas, do Canadá [Kobelt *et al.,* 2006] e da Alemanha [Zink *et al.,* 2006], apontam porém para consumos de 16-18 consultas gerais e de 3,7-4,2 consultas de reumatologia/ano. Estes resultados não devem ser extrapoláveis pois existem múltiplos fatores que podem influenciar os resultados obtidos em cada país - o próprio sistema de saúde, as características das populações estudadas, as características dos doentes (incluindo a presença de co-morbilidades médicas e gravidade da doença) e os métodos de determinação da utilização dos cuidados de saúde. Independentemente destes aspetos, os vários estudos documentam um aumento da utilização dos serviços de saúde, havendo múltiplas razões para isso:

a) A utilização dos cuidados primários motivada pelos sintomas diretamente relacionados com a SpA/EA (dor lombar, dor articular, entesite) e/ou a presença de co-morbilidades e/ou relacionado com complicações associadas ao tratamento;

b) Recurso a cirurgias por problemas diretamente relacionados com SpA/EA (ex. artroplastia da anca ou joelho) e/ou relacionadas com situações de co-morbilidade;

c) Recurso a consultas de reumatologia refletindo o normal seguimento dos doentes para ajuste e monitorização da terapêutica;

d) Recurso a consultas de outras especialidades como gastrenterologia, nefrologia e cardiologia, por problemas relacionados com a própria SpA/EA ou complicações terapêuticas.

Neste âmbito importa também analisar o impacto da EA em termos de custos. Os custos totais anuais ascendem a $6,720 dólares (dados de 1999; mediana de $1,495) dos quais 73,6%

representem custos indiretos e 26,4% custos diretos. Curiosamente, apenas 39% dos doentes contribuem para os custos indiretos [Ward, 2002] sendo a incapacidade funcional o principal determinante desses custos. A probabilidade de ter um custo total elevado aumenta 3 vezes por cada elevação de 1 ponto no "*Health Assessment Questionnaire disability index modified for the spondylarthropathies*" (HAQ-S; oscila entre 0-3) [Ward, 2002]. Assim, as intervenções que possam contribuir para manter ou melhorar a capacidade funcional dos doentes poderão ter um grande potencial para reduzir os custos associados à EA. As co-morbilidades poderão também exercer influência mas são necessários mais estudos para avaliar o seu impacto, em termos de número e de gravidade, no consumo de recursos e custos associados [Boonen *et al.*, 2002].

Tendo em consideração os aspetos da prevalência, da progressiva e irreversível anquilose das articulações afetadas, das co-morbilidades e do impacto socioeconómico da doença parece evidente que o diagnóstico numa fase inicial e a introdução atempada de um tratamento efetivo deverão exercer uma influência positiva no prognóstico da doença - **o que representa o atual paradigma na abordagem da doença.** Vários estudos mostraram existir um atraso diagnóstico superior a 8 anos, entre o início dos sintomas e o diagnóstico, motivando um atraso do início da terapêutica [Feldtkeller *et al.*, 2003]. Este é um período crítico para a ocorrência de dano estrutural. A resposta às diferentes terapêuticas é porém imprevisível, mesmo em doentes clinicamente semelhantes.

A introdução de terapêuticas biológicas tendo como alvo o TNF alfa - infliximab, etanercept, adalimumab, golimumab – e a iminente introdução de outras com diferentes alvos terapêuticos, modificaram/-rão a prática clínica com prováveis benefícios no controlo da sintomatologia e provavelmente em termos de prognóstico. Os critérios para classificar

doentes com EA, nomeadamente os critérios modificados de Nova Iorque [van der Linden *et al.*, 1984a], requerem a presença de sacroiliite radiológica. Foi estimado um período de 6 a 8 anos entre o início da inflamação nas articulações SI e a sua possível deteção nas radiografias convencionais [Rudwaleit *et al.*, 2005, 2009a; Bennett *et al.*, 2008]. Embora desconheçamos a proporção de doentes com SpA que evoluem para EA, os doentes com SpA axial não-radiográfica têm parâmetros de atividade de doença semelhante aos doentes com EA estabelecida em termos de sinais e sintomas [Rudwaleit *et al.*, 2009a], pelo que se colocou a hipótese de que poderão corresponder a diferentes estádios da mesma doença [Rudwaleit *et al.*, 2005]. Assumindo esta hipótese como verdadeira, os novos critérios ASAS para SpA axial, podem contribuir para uma mais rápida e precoce identificação da doença numa fase anterior ao aparecimento das alterações radiográficas.

A avaliação da eficácia da terapêutica tem sido feita com recurso ao BASDAI 50 [Braun *et al.*, 2003] e/ou critérios de melhoria ASAS [Anderson *et al.*, 2001], embora se desconheça em que medida são realmente adequados para esse fim. A recente introdução do ASDAS revelou-se como um instrumento muito útil para a prática clínica, pelo elevado poder discriminativo na avaliação da atividade da EA e na resposta à terapêutica [Lukas *et al.*, 2009; van der Heijde *et al.*, 2009; Machado *et al.*, 2011]. Não tem, no entanto, qualquer papel como preditor de resposta à terapêutica.

Neste contexto, a identificação de novos marcadores com maior poder discriminativo, passíveis de serem aplicados para se estabelecer o diagnóstico, o prognóstico e como preditores de resposta à terapêutica, assume grande relevância. Ao facilitar a abordagem do doente e simultaneamente ao possibilitar uma melhor adequação da terapêutica poderiam contribuir para uma racionalização dos custos.

Nos últimos anos, o progresso dos métodos de genotipagem e do desenho dos próprios estudos de investigação, revolucionaram o conhecimento das doenças comuns tendo tido grande impacto na EA. Um enorme salto foi dado entre 2007 e 2011 conhecendo-se atualmente 14 genes para além do HLA-B, associados à doença [Brown *et al.*, 2010; Evans *et al.*, 2011]. Dois estudos recentes, puseram ainda em evidência que o tratamento com anti-TNF α leva a alterações significativas da expressão génica e no perfil proteico, o que faz com que estes métodos possam provavelmente contribuir para uma melhor compreensão dos mecanismos patogénicos envolvidos [Visvanathan *et al.*, 2008; Haroon *et al.*, 2010] bem como para a identificação de marcadores de resposta à terapêutica. Os marcadores atualmente disponíveis associados a favorável resposta à terapêutica são a idade jovem, a positividade para o HLA-B27, a elevação dos reagentes de fase aguda (CRP) e a presença de sinais de inflamação na RMN. De forma inversa, a idade avançada, a presença de alterações estruturais e de alterações funcionais são preditores de ausência ou má resposta [Rudwaleit *et al.*, 2004, 2008]. Embora sejam biomarcadores com significância estatística parece claro que novos biomarcadores mais discriminativos são necessários para serem usados na prática clínica. Este foi o grande desafio deste trabalho e representa um enorme desafio para o futuro próximo. Para a prática clínica, a integração de novos biomarcadores com os novos critérios de classificação e de monitorização poderá mostrar-se muito útil para clínicos, doentes e sociedade.

2. COMPONENTE CIENTÍFICA

A realização deste projeto de investigação motivou a criação de um grupo de trabalho denominado **CO**nhcer a **R**ealidade **POR**tuguesa sobre **E**spondilite **A**nquilosante (CORPOREA). Nele participaram inúmeros médicos de vários centros de Reumatologia (e um de Fisiatria) nacionais - Centro Hospitalar de Lisboa Ocidental, Hospital de Egas Moniz EPE, Lisboa; Centro Hospitalar de Lisboa Norte, Hospital de Santa Maria EPE, Lisboa; Instituto Português de Reumatologia, Lisboa; Hospital Militar Principal, Lisboa; Hospital Curry Cabral EPE; Hospital Garcia de Orta EPE, Almada; Centro Hospitalar do Alto Minho, Hospital Conde de Bertiandos EPE, Ponte de Lima; Hospital de São Marcos, Braga; Hospital de Faro EPE, Faro; Centro Hospitalar Baixo Vouga, Hospital Infante D. Pedro EPE, Aveiro; Centro Hospitalar Oeste Norte, Centro Hospitalar das Caldas da Rainha EPE, Caldas da Rainha. Por se pretender reduzir possíveis fatores de enviezamento, relacionados com o recrutamento de doentes, não foram incluídos os centros de Reumatologia das regiões autónomas. Foram ainda estabelecidas parcerias nacionais (Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa) e internacionais (Diamantina Institute for Cancer, Immunology and Metabolic Medicine, Princess Alexandra Hospital, University of Queensland, Brisbane, Australia; Institut Cochin, Department of Immunology and INSERM, Paris, França), o que permitiu reunir as condições necessárias à realização dos vários tópicos do projecto.

O envolvimento das múltiplas unidades nacionais, permitiu oferecer uma dimensão nacional ao projecto. Num período de 6 meses, uma equipa de médicos do Hospital Egas Moniz dirigiu-se periodicamente aos diversos centros para entrevistar, avaliar e colher amostras biológicas dos doentes recrutados em cada local. Foi assim constituída uma cohorte de 369

doentes, com o diagnóstico de EA de acordo com os critérios modificados de Nova Iorque, com idade superior a 18 anos, de ascendência Portuguesa até à segunda geração, que manifestaram a sua concordância em participar neste projeto de investigação, tendo para isso assinado de livre vontade o termo de responsabilidade. O projeto de investigação obteve o parecer favorável da Comissão de Ética do CHLO e de todos os estabecimentos hospitalares envolvidos. Paralelamente, recrutaram-se em diferentes locais, amostras biológicas de indivíduos saudáveis que constituíram o grupo de controle. Foram ainda selecionados de forma aleatória alguns indivíduos que integram a base nacional de dadores de medula óssea do Centro de Histocompatibilidade do Sul.

Foram objetivos deste Projecto:

a) Caracterização clínica e epidemiológica da EA em Portugal;

b) Validação para língua Portuguesa dos principais índices de Bath;

c) Criação de instrumentos para monitorização da doença;

d) Identificação de biomarcadores com utilidade para o diagnóstico e avaliação prognóstica da doença.

Os principais resultados do estudo efetuado serão sumariamente descritos nos tópicos seguintes, com referência aos artigos em que foram publicados.

2.1. INVESTIGAÇÃO CLÍNICA

2.1.1. Versão Portuguesa dos Índices de Bath

Pimentel-Santos FM, Pinto LT, Santos H, Barcelos A, Cunha I, Branco JC, Ferreira PL. **Portuguese version of the bath indexes for ankylosing spondylitis patients: a cross-cultural adaptation and validation.** *Clin Rheumatol.* 2012 Feb; 31(2): 341-6.

Foram adaptados e validados para Português (falado em Portugal), o "The Bath Ankylosing Spondylitis Disease Activity Index" (BASDAI), o "The Bath Ankylosing Spondylitis Functional Index" (BASFI), o "The Bath Ankylosing Spondylitis Metrology Index" (BASMI) e o "The Bath Ankylosing Spondylitis Global Score" (BASG), que constituem índices frequentemente utilizados na avaliação dos doentes na prática clínica corrente. As versões Portuguesas destes índices encontram-se no Anexo II. Após tradução e retroversão dos questionários, foi feita a sua aplicação a 78 doentes, com o intuito de se avaliar a Consistência Interna, a Reprodutibilidade e a Validade dos mesmos. A Consistência Interna oscilou entre 0,747 e 0,953. Os Coeficientes de Correlação obtidos na prova do teste-reteste foram de 0,875/ 0,937/ 0,831/ 0,961 para o BASDAI, BASFI, BASMI e BASG, respetivamente. Todos os índices mostraram ser compreensíveis de acordo com a opinião expressa por um painel de doentes. Os vários aspetos da validade foram também assegurados. Este estudo teve como principal limitação a não inclusão de medidas de resposta ao tratamento, pelo que a determinação da sensibilidade à mudança não foi determinada.

As versões Portuguesas destes índices mostraram possuir propriedades clinimétricas idênticas às versões originais e sobreponíveis às versões validadas para outros idiomas, pelo que

poderão ser utilizadas para a correta avaliação dos doentes, que usam o Português falado correntemente em Portugal, na prática clínica corrente ou para fins de investigação.

2.1.2. Caracterização Clínica e Epidemiológica da EA em Portugal

Pimentel-Santos FM, Mourão AF, Ribeiro C, Costa J, Santos H, Barcelos A, Pinto P, Godinho F, Cruz M, Sousa E, Santos RA, Rabiais S, Félix J, Fonseca JE, Guedes-Pinto H, Brown MA, Branco JC and *CORPOREA* Study Group. **Spectrum of ankylosing spondylitis in Portugal. Development of BASDAI, BASFI, BASMI and mSASSS reference centile charts.** *Clin Rheumatol*. 2012 Mar; 31(3): 447-54.

No grupo total dos 369 doentes avaliados, 62,3% eram do sexo masculino conferindo um *ratio* masculino:feminino de 1,6:1. A idade média±(DP) do grupo era de 45,4±13,2 anos (oscilando entre 20-79 anos) com uma duração média de doença de 11,4±10,5 anos (oscilando entre 0-46 anos). A idade média de início dos sintomas foi de 26,5±10,8 anos e a idade média do diagnóstico de 34,1±13,4 anos [o início juvenil (idade < 16 anos) foi reportado em 39 (10.6%) dos casos e o início tardio (idade > 40 anos) em 37 (10%)], o que denota um atraso no estabelecimento do diagnóstico de 7,6±9,0 anos [inferior a 1 ano em 51 (13.8%) casos e superior a 10 anos em 86 (23.3%)]. Atrasos diagnósticos idênticos foram descritos noutros países [Feldtkeller *et al.*, 2003]. Reportaram história familiar em primeiro grau 17.6% dos doentes. A dor lombar (42,3%) foi a manifestação inicial mais comum. No momento da avaliação, 49,9% apresentavam um envolvimento axial, 2.4% um envolvimento periférico, 40.9% misto e 7.1% entesopático isolado. Referiram manifestações extra-articulares 35,2% dos doentes tendo sido a uveíte anterior aguda (33.6%) a mais comum. A psoríase (6.2%), a doença intestinal inflamatória (2.4%), a doença pulmonar (1.4%), a doença cardíaca (1.1%) e a renal (0.3%) foram descritas de forma menos frequente.

A avaliação global da doença efetuada pelo doente e pelo médico (4.7 *vs* 2.6, p<0.001) mostraram ser significativamente diferentes, não considerando os médicos a doença tão grave quanto os doentes. O valor médio do BASDAI foi de 4,2±2,3, do BASFI de 4,1±2,7, do BASMI 4,0±2,5 e do mSASSS 20,9±23,1.

A abordagem terapêutica foi também analisada, considerando-se para esse efeito, a medicação efetuada no momento da avaliação. A maioria dos doentes encontrava-se sob terapêutica com AINE's (79.1%), de forma contínua ou intermitente e uma pequena mas significativa proporção, tomava corticoides (17.6%). Estes resultados confirmam valores encontrados, em estudos prévios, na nossa população [Sousa *et al.,* 2008]. Estavam sob terapêutica DMARDs 48.5% dos doentes, sendo a SSZ (30.9%) o mais utilizado, seguida pelo MTX (8.4%); a associação SSZ e MTX eram cumpridas por 6% dos doentes. Sob terapêutica anti-TNF-α encontravam-se 22% dos doentes. Este valor sendo semelhante ao descrito noutras populações [Collantes *et al.,* 2007; Strömbeck *et al.,* 2009], é inferior ao estimado por vários líderes de opinião (30-49%) [Landewe *et al.,* 2004; Pham *et al.,* 2006] como sendo a percentagem de doentes que carecem deste tipo de terapêutica. A correlação positiva entre os valores de BASMI e a terapêutica anti-TNFα (p=0.024) pode, por outro lado, ser uma evidência indireta de que estes fármacos são iniciados tardiamente no curso da doença.

Tal como referido noutras populações, a EA afeta mais frequentemente os indivíduos do sexo masculino. A idade de início de sintomas, a idade de diagnóstico e o atraso no diagnóstico são porém, idênticas nos dois sexos, na nossa população. Verificou-se, no entanto, que nas mulheres os valores médios do BASDAI e do BASFI eram 1,2 e 0,7 pontos superiores, respetivamente, e que os do BASMI e do mSASSS eram 0,8 e 17,6 pontos inferiores, respetivamente, sendo as diferenças estatisticamente significativas. Estes resultados parecem

apontar para que a doença tenha uma maior atividade (BASDAI) e maior repercussão funcional (BASFI), com melhor metrologia (BASMI) e melhor compromisso radiológico (mSASSS) nas mulheres comparativamente aos homens. Estas diferenças são consistentes quando os resultados são avaliados como um todo ou, quando diferentes períodos de tempo, são considerados. Esta noção é reforçada pela análise das tabelas de percentis elaboradas para os índices de BASDAI, BASFI, BASMI e mSASSS, com base em "*generalized linear models*". Apesar das diferentes metodologias estatísticas utilizadas, da pequena dimensão da amostra, dos diferentes *ratios* sexo masculino:femininos, os resultados da população Portuguesa foram muito semelhantes aos reportados na população Inglesa [Taylor *et al*., 1998]. Estes gráficos fornecem informação sobre a atividade da doença e o seu impacto em termos de repercussão funcional, metrológica e radiológica ao longo do tempo. Podem assim, permitir comparar uma mesma população em diferentes momentos ou diferentes populações. Este tipo de representação visual pode ainda, ao contribuir para uma melhor compreensão da doença, aumentar a *compliance* do doente à intervenção proposta. A sua aplicação numa base individual na prática clínica corrente carece, no entanto, de uma adequada validação o que implica a recolha prospetiva de dados.

Em conclusão, neste tópico procedeu-se à validação para língua Portuguesa dos principais índices de *Bath* utilizados na clínica. Paralelamente, promoveu-se a caracterização clínica e demográfica dos doentes com EA em Portugal. O desenvolvimento das tabelas de percentis constitui de forma adicional, um instrumento útil para avaliar modificações do padrão da doença ao longo do tempo e/ou em resposta às intervenções terapêuticas efetuadas, bem como para estabelecer comparações com outros grupos populacionais.

2.2. ESTUDOS DE BASE GENÉTICA

2.2.1. Estudos de Genes MHC (HLA classe I, II e III)

Pimentel-Santos FM, Matos M, Ligeiro D, Mourão AF, Ribeiro C, Costa J, Santos H, Barcelos A, Pinto P, Godinho F, Cruz M, Sousa E, Santos RA, Fonseca JE, Trindade H, Guedes-Pinto H, Brown MA, Branco JC and *CORPOREA* Study Group. **HLA Class I and II Associations of Ankylosing Spondylitis.** (Submitted to *J Rheumatol*.)

Sousa E, Caetano-Lopes J, Pinto P, Pimentel F, Teles J, Canhão H, Rodrigues A, Resende C, Mourão AF, Ribeiro C, Pinto TL, Rosa CM, da Silva JA, Branco J, Ventura F, Queiroz MV, Fonseca JE. **Ankylosing spondylitis susceptibility and severity-contribution of TNF gene promoter polymorphisms at positions -238 and-308.** *Ann N Y Acad Sci*. 2009 Sep; 1173: 581-8.

Na *cohort* Portuguesa, 285:355 (80.3%) dos doentes com EA eram *HLA*B27* positivos. Este resultado é similar ao da Grécia (80.5%) [Alamanos *et al.*, 2004], inferior ao de Espanha (94.3%) [Fernández-Sueiro *et al.*, 2004] mas superior ao valor encontrado na Turquia (70%) [Gunal *et al.*, 2008].

Um grupo aleatório, de doentes *HLA*B27* positivos (n=188) e de controlos (n=189) foram selecionados e genotipados para as classes I e II do HLA por PCR-SSOP. Os haplótipos estendidos foram estimados por *Expectation Maximization algorithm* usando o *software Arlequin v3.11*. As comparações caso-controlo foram feitas com tabelas de contingência para os *loci* e para os haplótipos estimados. As associações entre as características genéticas e fenotípicas foram efetuadas através de regressões lineares e logísticas binomiais usando o *software* Stata10.1.

Na população Portuguesa não se encontraram associações dos *loci* HLA estudados (para além do *HLA*B27*) e a suscetibilidade para a doença. Esta situação contrasta com os resultados de

estudos prévios, em que vários alelos classe I e II foram descritos como associados, positivamente ou negativamente à doença. Na classe I, o *HLA-B60 (B*40)* e o *HLA-B*14*, foram descritos como alelos associados à suscetibilidade para a EA em populações Caucasianas [Robinson *et al.,* 1989] e Africanas (Togo e Camarões) [Merino *et al.,* 2005; Brown *et al.,* 2008], respetivamente. O *HLA-B*07* [Kchir *et al.,* 2010] e o *HLA-B*51*, pelo contrário, parecem exercer proteção para a EA em várias populações Mediterrânicas [Hajjej *et al.,* 2006; Kchir *et al.,* 2010]. Na classe II, o *HLA-DR*01* foi descrito como conferindo suscetibilidade para a EA, independentemente do *HLA-B*27*, na população Inglesa e Mexicana [Brown *et al.,* 1998; Vargas-Alarcon *et al.,* 2002], o *HLA-DR*08* na Inglesa [Brown *et al.,* 1998] e o *HLA-DQ*02* na Espanhola [Sanmarti *et al.,* 1987].

Na população estudada identificaram-se porém, várias associações entre alelos e as características fenotípicas da doença. O *HLA-B*18* associou-se a início da doença em idade jovem, o *DQB1*02* a história familiar, o *B*14, B*44, DRB1*08* a AAU, o *B*14* e o *DRB1*08* a gravidade radiológica. Em contraste, o alelo *DQB1*04* parece conferir proteção em termos de atividade e de repercussão funcional, metrológica e radiológica da doença. Nenhuma destas associações foi previamente reportada na literatura com exceção da associação entre *HLA-DRB1*08* e AAU [Brown *et al.,* 2008]. Os diferentes resultados obtidos nos vários estudos poderão ser atribuídos a diferentes metodologias e a diferenças étnicas existentes.

Atendendo à densidade de genes e à frequência dos desequilíbrios de ligação nesta região a identificação de variantes realmente associadas à EA torna-se muito difícil. O estudo dos haplótipos pode facilitar a identificação dessas variantes pelo que se procedeu a essa análise. O haplótipo *A*02/B*27/Cw*02/DRB1*01/DQB1*05* (p<0,0001; OR=39,06; CI 95% [2,34-651]) foi o único identificado como sendo capaz de conferir suscetibilidade para a EA. Pelo

contrário, foram encontrados vários haplótipos associados às características fenotípicas da doença. A associação mais robusta estabeleceu-se com o *A*02/B*27/Cw*01/DRB1*08/DQB1*04,* que parece conferir proteção para a atividade e repercussão funcional e radiológica da doença. A inexistência na literatura de estudos semelhantes (envolvendo haplótipos estendidos) torna difícil estabelecer comparações com outras populações.

O estudo efetuado sendo inovador apresenta algumas limitações relacionadas com a pequena dimensão da amostra e pela inclusão estrita de doentes HLA-B27 positivos. Os resultados obtidos carecem assim de confirmação em estudos futuros.

Da parceria estabelecida com o Instituto de Medicina Molecular obtiveram-se novos dados relacionados com a avaliação da influência dos polimorfismos, nas posições -238 e -308, do promotor do gene *TNF* (classe III do HLA) na suscetibilidade e prognóstico da EA. Os SNPs foram analisados por "*restriction fragment length polymorphisms* " (RFLP) em doentes (n=141) e controlos (n=117).

Constatou-se que os doentes com EA, apresentavam uma frequência do alelo A na posição -238, menor (10%) que os controlos (18%), sugerindo um efeito protetor deste alelo na suscetibilidade para a doença ($p<0.05$). A frequência dos alelos na posição -308 foi semelhante nos dois grupos, doentes e controlos. Estes resultados eram independentes da positividade para o HLA-B27. De forma adicional, o genótipo -238 GA/AA associou-se a valores elevados de VS comparativamente ao -238 GG (45.7±28.8mm/1ª hora *vs.* 20.2±15.3mm/1ª hora, $p<0.05$, respetivamente) e os doentes com genótipo -308 GA/AA tinham um início mais tardio da doença, comparativamente com os -308 GG (28±11 anos *vs.* 24±9 anos, $p<0,05$, respetivamente). Na literatura, encontram-se vários estudos com foco em

polimorfismos nas posições, −308 (G/A) e −238 (G/A) do promotor do gene do *TNF-α* [Verjans *et al.*, 1994; Fraile *et al.*, 1998b; Hohler *et al.*, 1998; Kaijzel *et al.*, 1999; McGarry *et al.*, 1999; Martinez-Borra *et al.*, 2000; Milicic *et al.*, 2000; Gonzalez *et al.*, 2001; Vargas-Alarcon *et al.*, 2006; Shiau *et al.*, 2007; Lu *et al.*, 2008; Chatzikyriakidou *et al.*, 2009; Nicknam *et al.*, 2009; Sousa *et al.*, 2009], com resultados discordantes. As pequenas dimensões das amostras, o insuficiente poder estatístico, a heterogeneidade clínica e as diferenças étnicas, têm sido apontados como possíveis fatores explicativos pelo que foram recentemente efetuadas duas meta-análises [Lee & Song, 2009; Li *et al.*, 2010]. Em nenhuma delas se verificou associação entre os polimorfismos estudados e a suscetibilidade para a EA, quando se considerou o grupo total ou apenas os doentes *HLA-B27* positivos.

Em conclusão, estimou-se uma prevalência baixa/moderada de HLA-B*27 (80.3%) na população Portuguesa com EA. Foi descrito um haplótipo capaz de conferir suscetibilidade para a doença e outro que de forma consistente se associa a proteção, em termos de atividade e gravidade da doença. O estudo dos polimorfismos nas posições -238 e -308 do promotor do gene do TNF, apontou para que os mesmos exerçam uma pequena influência na susceibilidade e prognóstico da EA.

2.2.2. Estudos de Genes não-MHC (IL23R, ERAP1, ANKH, TNFSF)

Pimentel-Santos FM, Ligeiro D, Matos M, Mourão AF, Sousa E, Pinto P, Ribeiro A, Sousa M, Barcelos A, Godinho F, Cruz M, Fonseca JE, Guedes-Pinto H, Trindade H, Evans DM, Brown MA, Branco JC. **Association of IL23R and ERAP1 genes with ankylosing spondylitis in a Portuguese population.** *Clin Exp Rheumatol*. 2009;27(5):800-6.

Pimentel-Santos FM, Ligeiro D, Matos M, Mourão AF, Sousa EV, Pinto P, Ribeiro A, Santos H, Barcelos A, Godinho F, Cruz M, Fonseca JE, Guedes-Pinto H, Trindade H, Brown MA, Branco JC and *CORPOREA* Study Group. ***ANKH* and Susceptibility to and Severity of Ankylosing Spondylitis.** *J Rheumatol* 2012;39(1):131-4..

Zinovieva E, Kadi A, Letourneur F, Cagnard N, Izac B, Vigier A, Said-Nahal R, Elewaut D, de Vlam K, Pimentel-Santos F, Chiocchia G, Breban M. **Systematic candidate-gene investigations in the SPA2 locus (9q32) show an association between the gene TNFSF8 and susceptibility to spondyloarthritis.** *Arthritis Rheum.* 2011;63(7):1853-9.

A associação entre a EA e os genes, *ERAP1* e *IL23R*, tinha sido recentemente reportada nas populações Norte-Americana e Inglesa, com riscos atribuídos de 25% e 9%, respetivamente [WTCCC/TASC, 2007]. Outros estudos prévios mostravam resultados contraditórios em relação ao gene *ANKH* [Tsui *et al.*, 2005; Timms *et al.*, 2007; Furuichi *et al.*, 2008;].

A associação da *IL23R* com a EA tinha sido replicada na população do Canadá [Rahman *et al.*, 2008] e Espanhola [Rueda *et al.*, 2008]. Outros estudos demonstraram ainda a associação dos SNPs da *IL23R* com a suscetibilidade para a doença de Crohn's (CD) [Barrett *et al.*, 2008], psoríase [Cargill *et al.*, 2007] e artrite psoriásica [Filer *et al.*, 2008; Liu *et al.*, 2008; Huffmeier *et al.*, 2009], pelo que este gene parece conferir suscetibilidade para as espondilartrites sero-seronegativas como um todo e assim se pode explicar, pelo menos de forma parcial, a sua coocorrência. Em contraste, o *ERAP1* parece conferir suscetibilidade de forma específica para a EA. O *ANKH*, apesar dos resultados contraditórios em termos de associação à EA, revestia-se de particular interesse porque ratinhos, com mutações com perda

de função do gene homólogo, *ank*, desenvolviam uma mineralização ectópica e uma anquilose óssea muito semelhante à da EA [Ho *et al.*, 2000]. Em Humanos, a descrição de casos de condrocalcinose associados a mutações com ganho de função [Williams *et al*., 2002; Zhang *et al*., 2005] e de vários casos de espondiloartropatia numa família associados a homozigotia para uma mutação com perda de função do gene [Morava *et al*., 2010] reforçavam o interesse colocado neste gene.

Neste contexto, pretendeu-se testar na população Portuguesa, a associação entre estes SNPs e a suscetibilidade para a doença e/ou a possível influência nas suas manifestações clínicas. A genotipagem das variantes alélicas da *IL23R*, *ERAP1*e *ANKH* foi realizada por "*TaqMan allelic discrimination assays*".

Foram analisados 5 SNPs para o *ERAP1*, 1 para *LN-PEP*, 8 para a *IL23R* e 4 para o *ANKH*. Os SNPs rs27044 e rs30187 do *ERAP1* e os rs1004819 e rs10889677 da *IL23R* mostraram significativa associação à doença. Confirmou-se assim a associação destes genes à doença na população Portuguesa, com riscos atribuídos de 9,7% e 11%, respetivamente. Não se verificou porém, associação com o SNP do *LN-PEP*, que flanqueia o *ERAP1*, e que tinha mostrado associação com a EA na população Inglesa [WTCCC, 2007]. Do mesmo modo, não se estabeleceu associação com nenhum dos 4 SNPs estudados do *ANKH*, sugerindo que este gene não deve ser um determinante major da suscetibilidade para a EA na nossa população. Finalmente, não se estabeleceu qualquer associação entre os diferentes SNPs estudados e as manifestações da doença como a idade de início de sintomas, BASDAI, BASFI, BASMI ou mSASSS.

Em conclusão, confirmou-se que os genes da *IL23R* e do *ERAP1* se encontram associados a suscetibilidade para a EA na população Portuguesa contribuindo com um risco de 11% e

9,7%, respetivamente, não se verificando o mesmo com o ANKH. Nenhum dos genes estudados pareceu exercer influência nas manifestações clínicas da doença.

Em parceria com o INSERM foi possível contribuir para a validação da associação de um SNP do *TNFSF8*, recentemente descrito, e a EA.

Na sequência de um "*genome-wide linkage study*" realizado na população Francesa foi identificada uma nova região associada a suscetibilidade para as SpA, no cromossoma 9q31-34, denominado SPA2 [Miceli-Richard *et al.*, 2004]. Esta região contém cerca de 85 genes, sendo curiosamente uma região paráloga do MHC, pelo que poderia conter outros fatores de suscetibilidade para as SpA [Said-Nahal *et al.*, 2002]. Foram selecionados nove genes para um estudo adicional: *ZNF618*, *A1L4R1_HUMAN* (AF495724), *AMBP*, *KIF12*, *ORM1*, *ORM2*, *C9ORF91*, *ENSESTG000000230601*, e *TNFSF8*. Após ter sido efetuado o estudo de confirmação, apenas um SNP, rs3181357, localizado no gene *TNFSF8* mostrou uma associação significativa com a doença. Este SNP foi posteriormente replicado usando doentes Portugueses e Belgas, tendo-se obtido um resultado muito significativo (OR=2.14; p= 0.0001).

O *TNFSF8*, pode ser assim considerado um gene candidato com relevância em termos funcionais. Codifica o CD30L (CD153), um ligando da superfamília TNF expresso em células CD4+ T ativadas, células apresentadoras de antigénio e neutrófilos, que interagem com o recetor CD30 em células Th efetoras ou de memória. A via de sinalização CD30L/CD30 tem um papel crítico na diferenciação de células Th17 *in vitro* e em *in vivo* [Sun *et al.*, 2010]. É possível que o SNP do *TNFSF8* descrito possa estar diretamente implicado, de forma isolada ou em combinação, com outros polimorfismos situados na mesma região, na predisposição para as SPA. Em alternativa, pode mascarar uma ou mais variantes não identificadas no

estudo, devido provavelmente a mecanismos de desequilíbrio de ligação, que exerçam esse efeito para suscetibilidade destas doenças. Algumas dúvidas se levantam no entanto, acerca do papel real desta variante pelo facto de não ter sido identificada nos GWAS efetuados no contexto do WTCCC/TASC.

2.3. ESTUDOS DE EXPRESSÃO GÉNICA

2.3.1. Análise pela tecnologia de microarrays

Pimentel-Santos FM, Ligeiro D, Matos M, Mourão AF, Costa J, Santos H, Barcelos A, Godinho F, Pinto P, Cruz M, Fonseca JE, Guedes-Pinto H, Branco JC, Brown MA, Thomas GP. **Whole blood transcriptional profiling in ankylosing spondylitis identifies novel candidate genes that might contribute to the inflammatory and tissue-destructive disease aspects.** *Arthritis Res Ther*. 2011 Apr 7; 13(2): R57.

Os estudos de associação genética permitiram identificar vários genes que contribuem para a suscetibilidade da EA mas este tipo de abordagem fornece pouca ou nenhuma informação relacionada com a alteração da atividade génica que ocorre durante a evolução da doença. Os perfis de transcrição criam uma "fotografia" da atividade celular num determinado momento e podem fornecer informação relevante sobre os mecanismos moleculares envolvidos na doença. O objetivo *major* na realização deste estudo foi tentar avaliar em que medida os perfis de expressão genómica, podem ser úteis na distinção entre doentes e controlos e na identificação de vias fisiopatológicas com relevância na patogenia da EA.

No estudo realizado, de identificação e confirmação, foram envolvidos 18 doentes com EA e 18 controlos emparelhados de acordo com o sexo e a idade (± 5 anos). Foram apenas incluídos doentes com BASDAI>4 e BASFI>4, medicados com AINE's e/ou SSZ e excluídos

doentes sob anti-TNF, corticoides ou metotrexato. Para o estudo de validação foram envolvidos 78 doentes e 78 controlos emparelhados para sexo e idade. No estudo de identificação foram usados *microarrays* com recurso ao "*Human HT-12 V3 Expression BeadChips*"(Illumina, CA). No estudo de confirmação e validação recorreu-se a um método de PCR quantitativa em tempo real denominado "*TaqMan Low Density Array Cards®*" (TLDA). Foram identificados 221 genes diferencialmente expressos entre doentes e controlos com $p<0,0005$. Dos 47 genes selecionados para o estudo de validação (de acordo com os níveis de expressão, valor de *p* e relevância biológica), apenas 14 foram de facto validados. Entre eles, encontram-se genes com um papel bem documentado nos mecanismos de inflamação e outros que contribuem para uma melhor compreensão dos mecanismos envolvidos na progressão da EA.

O *PTPN1* e o *DOCK10*, estão ambos envolvidos nas ações mediadas pela IL4 [Paul & Ohara, 1987], o que é de particular interesse uma vez que tem um papel relevante na patogenia da EA. A proteína tirosina fosfatase 1B (PTP1B) é uma enzima expressa de forma ubiquitaria, que regula negativamente múltiplas vias de sinalização dependentes da fosforilação da tirosina, incluindo a via de sinalização da IL4 [Lu *et al.*, 2008]. A Dock10 é regulada pela IL4 em células B [Yelo *et al.*, 2008].

A IL4 é um produto dos linfócitos T ativados, tendo efeitos de estimulação e de inibição das células B e T [Paul & Ohara, 1987, O'Garra *et al.*, 1988; Jelinek & Lipsky, 1988; Rousset *et al.*, 1988] e provavelmente também influencia a produção de células CD8+ T. Foi demonstrado recentemente que alguns subtipos destas células produzem 'Th2' citoquinas como a IL4, IL5 e IL10, embora a produção de citoquinas Th1, como o IFNγ seja a sua função habitual [Baek *et al.*, 2008]. O potencial papel da IL4-na produção de células CD8+ T

não está completamente esclarecido mas os subtipos de células T, CD8+/TCR αβ+, com uma função regulatória (expressando CD25+, CTLA4+, Foxp3+, e sendo negativas para o IFNγ e perforina), foram previamente descritas no sangue periférico de doentes com EA [Jarvis *et al.*, 2005]. Estes resultados foram confirmados em estudos recentes que sugeriram um padrão de diferenciação alterado de células T, em doentes com EA e em saudáveis *HLA-B27*+ [Zhang *et al.*, 2009]. É assim possível que esta predisposição para gerar células T, IL4+CD8+ possa ter algum efeito na patogenia das SpA [Jarvis *et al.*, 2005; Zhang *et al.*, 2009].

O *SPOCK2*, também conhecido como Sparc/osteonectina, foi implicado, num estudo recente, como discriminante entre SpA e controlos [Sharma *et al.*, 2009] e como exercendo um papel na regulação, produção, organização e manutenção da matriz cartilagínea [Hausser *et al.*, 2004; Gruber *et al.*, 2005]. Neste processo, o TGFβ e o IFNγ, exercem efeitos antagónicos na regulação da colagenase (*COL1A2*); o TGF β ativa a enzima (via de sinalização do Smad) e o IFNγ inibe-a (via sinalização do Stat1). Curiosamente, a proteína produzida pelo *EP300* faz parte do grupo de co-activadores de transcrição nuclear (nuclear p300/CBP transcriptional coactivators) que interagem com o Smad 3 e o Stat1a; da integração de sinais resulta a ativação ou inibição da *COL1A2* [Ghosh *et al.*, 2001]. A diminuição da expressão da *EP300* promove um estado pró-inflamatório [Ahmad *et al.*, 2007] que contribui para a degradação da cartilagem. De forma adicional, a proteína p300, controlada pela fosfoinositido-3 cinase (PI3K)/AKT, é um importante co-ativador transcricional da Sox9 [Cheng *et al.*, 2009], que modula a expressão do componente major da matriz extracelular - o agrecano. Finalmente, a *EP300* parece estar associada à via da *WNT*, um mediador chave na formação óssea e das alterações da cartilagem que ocorrem na osteoartrose [Velasco *et al.*, 2010].

Em conclusão, foi validado um perfil de expressão génica para a EA a partir do sangue periférico e identificados genes candidatos que contribuem para a compreensão dos mecanismos inflamatórios e das alterações do metabolismo ósseo e cartilagíneo que ocorrem durante a evolução da doença. Novos estudos são porém necessários, para confirmação destes resultados.

3. CONCLUSÕES

Neste trabalho de investigação foi possível caracterizar a EA em Portugal. A maior prevalência no sexo masculino (1,6:1), o atraso diagnóstico de cerca de 8 anos, o envolvimento predominantemente axial, a uveíte como principal manifestação extra-articular, a elevada atividade e gravidade da doença traduzida por *scores* de BASDAI, BASFI e BASMI superiores a 4, a utilização generalizada de AINE's e a baixa prescrição de agentes anti-TNFα, a positividade baixa/moderada para o HLA-B27, constituem, de forma sumária, os aspetos mais relevantes para a elaboração da imagem que a EA assume no nosso país.

A validação para língua Portuguesa do BASDAI, BASFI, BASG e BASMI, índices indispensáveis para a monitorização adequada dos doentes foi alcançada tendo sido difundida e estando atualmente a ser utilizados de forma corrente. A elaboração de tabelas de percentis para o BASDAI, BASFI, BASMI e mSASSS, torna possível estabelecer comparações dos nossos doentes em diferentes momentos do tempo e com outros grupos populacionais. É possível que após uma validação adequada possam também vir a ser utilizados numa base individual, o que representaria um meio adicional, de fácil entendimento por parte dos doentes, para sua correta monitorização.

Os estudos de natureza genética efetuados contribuíram para uma melhor compreensão dos mecanismos fisiopatológicos envolvidos na EA e permitiram a identificação de potenciais biomarcadores com interesse na prática clínica, em termos de diagnóstico e prognóstico (e de adequação terapêutica). De facto, apesar do MHC conferir cerca de 50% da predisposição para a doença, em grande medida associada ao HLA-B27, outros genes foram identificados. Dos genes estudados na população Portuguesa, validaram-se algumas variantes do ERAP1 e da IL23R, como estando associados à EA. A identificação de um polimorfismo do TNFSF8, na população Francesa, posteriormente validado na população Portuguesa e Belga, constitui um novo dado que deverá ser confirmado em estudos futuros. Outro ponto de particular interesse constitui a identificação de um perfil de expressão, que diferencia os doentes com EA dos indivíduos saudáveis.

Os recentes avanços no conhecimento contribuem para uma melhor compreensão dos mecanismos subjacentes ao desencadear da doença e sua evolução e a identificação de novos alvos terapêuticos que favorecem o desenvolvimento de novos fármacos. A integração futura de dados de natureza clínica, imagiológica e genética (com recurso a diferentes abordagens), poderá contribuir para a construção de algoritmos que permitam estabelecer mais facilmente o diagnóstico e prognóstico e favoreçam a seleção da terapêutica numa base individual, o que pode representar o início de uma medicina personalizada.

Estudos envolvendo populações de diferentes etnias, de dimensões adequadas, permitindo análises de vários subgrupos de interesse permitirão complementar e validar a informação existente. Com base nos resultados encontrados vários estudos estão desenhados no sentido de contribuir de forma ativa para a estruturação do conhecimento nesta área.

ANNEXE II – VALIDATION OF BATH INDICES IN PORTUGUESE

1. BASDAI

ÍNDICE DE ACTIVIDADE DE BATH PARA A ESPONDILITE ANQUILOSANTE

FAÇA UM TRAÇO EM CADA UMA DAS LINHAS QUE SE SEGUEM PARA INDICAR A SUA RESPOSTA A CADA PERGUNTA, RELATIVAMENTE À ÚLTIMA SEMANA

1 Como descreveria, em geral, **a fadiga / o cansaço** que tem sentido?

NENHUMA ——————————————— MUITO INTENSA

2 Como descreveria, em geral, a dor que tem tido **no pescoço, nas costas ou na anca**, devido à doença?

NENHUMA ——————————————— MUITO INTENSA

3 Como descreveria, em geral, a dor / o inchaço que tem tido nas articulações, com excepção do **pescoço, das costas e da anca**?

NENHUMA ——————————————— MUITO INTENSA

4 Como descreveria, em geral, o **desconforto** sentido quando toca ou carrega em zonas que doem?

NENHUMA ——————————————— MUITO INTENSA

5 Como descreveria, em geral, a intensidade da **rigidez matinal** que tem tido **desde que acorda**?

NENHUMA ——————————————— MUITO INTENSA

6 Quanto tempo dura a rigidez matinal desde que acorda?

0 horas — ½ — 1 — 1½ — 2 ou mais horas

2. BASFI

ÍNDICE FUNCIONAL DE BATH PARA A ESPONDILITE ANQUILOSANTE

FAÇA UM TRAÇO EM CADA UMA DAS LINHAS PARA INDICAR O SEU NIVEL DE CAPACIDADE PARA CADA UMA DAS SEGUINTES ACTIVIDADES, DURANTE A ÚLTIMA SEMANA

Nota: Uma ajuda técnica é um acessório que o ajuda a executar uma acção ou um movimento

EXEMPLO:

FÁCIL ├──────┼──────────────────────────────┤ IMPOSSÍVEL

1 Calçar meias ou meias-calças (*collants*) sem ajuda de alguém nem ajuda técnica (por exemplo, um dispositivo auxiliar para calçar meias.

FÁCIL ├──────────────────────────────────────┤ IMPOSSÍVEL

2 Dobrar-se para a frente pela cintura para apanhar uma caneta do chão sem ajuda técnica.

FÁCIL ├──────────────────────────────────────┤ IMPOSSÍVEL

3 Esticar-se para chegar a uma prateleira alta sem a ajuda de alguém, nem ajuda técnica (por exemplo, alguém dar uma mão).

FÁCIL ├──────────────────────────────────────┤ IMPOSSÍVEL

4 Levantar-se de uma cadeira sem braços sem usar as mãos ou qualquer outra ajuda.

FÁCIL ├──────────────────────────────────────┤ IMPOSSÍVEL

5 Partindo da posição de deitado/a de costas no chão, pôr-se de pé sem ajuda.

FÁCIL ├──────────────────────────────────────┤ IMPOSSÍVEL

6 Ficar de pé sem apoio durante 10 minutos, sem sentir desconforto.

FÁCIL ├──────────────────────────────────────┤ IMPOSSÍVEL

7 Subir 12-15 degraus sem usar o corrimão ou ajuda técnica. **Um pé em cada degrau.**

FÁCIL ├──────────────────────────────────────┤ IMPOSSÍVEL

8 Olhar por cima do ombro sem virar o corpo.

FÁCIL ├──────────────────────────────────────┤ IMPOSSÍVEL

9 Fazer actividades fisicamente exigentes (por exemplo, exercícios de fisioterapia, jardinagem ou desporto).

FÁCIL ├──────────────────────────────────────┤ IMPOSSÍVEL

10 Executar as actividades diárias, em casa ou no trabalho.

FÁCIL ├──────────────────────────────────────┤ IMPOSSÍVEL

3. BASG

Bath Ankylosing Patient Global Score (BASG)

1) Por favor coloque uma cruz **(X)** sobre a linha abaixo para indicar o efeito que a sua doença tem tido no seu bem-estar durante a **ÚLTIMA SEMANA.**

muito bem muito mal

2) Por favor coloque uma cruz **(X)** sobre a linha abaixo para indicar o efeito que a sua doença tem tido no seu bem-estar nos **ÚLTIMOS 6 MESES.**

muito bem muito mal

Referencia original:
Jones SD, Steiner SL, A Calin (1996). The Bath Ankylosing Spondylitis Patient Global score (BAS-G) British Journal of Rheumatology. 35:66-71

ANNEXE III - RESEARCH FELLOWSHIPS

a) Bolsa da Faculdade de Ciências Médicas/Universidade Nova de Lisboa, 2007

b) Bolsa de Investigação da Sociedade Portuguesa de Reumatologia/Schering-Plough, 2007

c) Bolsa de Investigação Individual da Fundação para a Ciência e Tecnologia (SFRH/BD/37360/2007), 2007

d) Projectos de Investigação/Centro de Estudos de Doenças Crónicas (CEDOC), 2009

e) Wyeth Lederle Portugal

f) Grupo Medinfar